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PHYSIOLOGICAL CHANGES OCCURRING IN THE BLOOD OF THE PACIFIC SALMON (*ONCORHYNCHUS TSHAWYTSCHA*) ACCOMPANYING SEXUAL MATURATION AND SPAWNING[1]

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ABSTRACT

A study was made of the changes occurring in a number of blood constituents during the spawning migration of the Pacific salmon (*O. tshawytscha*). The glucose content of the blood increased to about twice that found in salmon in the sea. Rarely was glucose found in the urine. The concentrations of sodium and potassium decreased, the latter to a marked degree. Cholesterol values rose during the migratory period, then fell at the time of spawning to concentrations lower than those found in sea salmon. Total proteins exhibited a pronounced diminution. Protein-bound iodine decreased progressively from the sea to the spawning grounds. Estimations of the number of red blood cells, hemoglobin content and packed cell volume showed fluctuations during the migration period but by the time of spawning the blood picture was in general like that of the sea salmon.

Accompanying the above changes was a progressive rise in the concentration of 17-hydroxycorticosteroids to high levels at the time of spawning. Detailed comparison with the characteristic physiological and histological features of Cushing's syndrome revealed many similarities between the spawning salmon and the human patient suffering from Cushing's syndrome.

THE spawning migration of the Pacific salmon is characterized by a sequence of biological phenomena which have been reported in only a few other vertebrates and these are all fishes. Before leaving the sea the salmon accumulates a large store of fat in the muscles and liver. When ready for its fluvial migration to the spawning grounds, it begins a fast which continues for the remainder of the fish's life—often a period of

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many months. During its journey up the river the salmon builds a large mass of gonadal tissue which is derived entirely from other body components. Then, following spawning these fish all die within a short period of time. Several other fishes are known to die following their first reproduction; the eel, the lampreys, the eulachon and a small group of so-called annual fishes (1). With the exception of a histological description of the alimentary tract of the sea lamprey (2) no physiological or histological studies on these fishes at sexual maturity have been reported.

In previous communications (3, 4, 5, 6) the occurrence of a series of histological changes in the various organs and tissues of the sexually maturing and spawning Pacific salmon have been described. These include increased activity of the pituitary, hyperplasia of the adrenal cortical tissue followed by terminal degeneration in both organs; deteriorative changes in the liver, spleen, thymus, kidney, thyroid and hypertrophy of the islands of Langerhans. With few exceptions the histological alterations occurring in the spawning salmon are similar to those found in experimental hyperadrenocorticism and in Cushing's syndrome and would appear to be due to the same cause since a marked rise in the concentration of plasma 17-hydroxycorticosteroids was found to occur during the spawning migration (7). It was because of these likenesses between the changes in migrating salmon and the well known manifestations of hyperadrenocorticism that the present study was undertaken.

MATERIALS AND METHODS

Source of fish.—King (Chinook) salmon taken at successive periods in their sexual cycle from complete immaturity in the sea to spawning and post-spanwing decline (spent), 75 in all, were examined as described in previous publications (4, 5, 6, 7).[2,3] In this study two runs of king salmon were investigated. The first group consisted of a spring run which enters the Sacramento River principally from February through April, and does not spawn until fall—late August through early October. Upon entering fresh water the gonads of these fish have barely begun to develop and are usually only in the early stages of maturation even after reaching the spawning beds, which in some instances are 300 miles from the sea. The length of time they are in fresh water, and starving, is estimated to be 5 to 6 months or more. During most of this period the salmon remain inactive in deep holes, near the spawning grounds. Since the lower end of Butte Creek, the tributary stream where the spawning spring-run salmon were obtained, becomes inac-

[2] For their generous help in providing salmon employed in this study, we are greatly indebted to Dr. Alex J. Calhoun, Chief of the Inland Fisheries Branch of the California Department of Fish and Game and to the following members of his staff: Mr. Leo Shaporaloo, Mr. Richard J. Hallock, Mr. Willis A. Evans, Mr. William Van Woert, Mr. James A. Hinze, Mr. John Riggs; also to Mr. Phil M. Roedel and Mr. Eldon P. Hughes of the Marine Resources Branch.

[3] Our sincere thanks also go to Mr. John Pelnar, Regional Manager, U. S. Fish and Wildlife Service, Coleman Station, Anderson, California, for supplying us with a number of spawning salmon.

TABLE 1. CHANGES IN THE VARIOUS BLOOD CONSTITUENTS OF PACIFIC SALMON AT SUCCESSIVE STAGES IN THE SEXUAL CYCLE. MALES AND FEMALES COMBINED

	Sea	Migrating		Spawning	
		Fall-run 1–2 months in river	Spring-run 2–3 months in river	Fall-run 2–3 months in river	Spring-run 5–6 months in river
17-OHCS μg/100 ml. plasma	11.8 (12)*	41.4 (7)	51.5 (14)	54.9 (36)	105 (14)
Glucose mg./100 ml. plasma	99 (14)	214 (10)	210 (14)	198 (24)	129 (12)
Sodium meq./L plasma	187 (11)	162 (3)	160 (14)	145 (13)	165 (11)
Potassium meq./L plasma	1.8 (11)	1.0 (3)	0.8 (14)	0.67 (13)	2.0 (11)
Cholesterol mg./100 ml. plasma	501 (21)	530 (10)	754 (21)	365 (17)	126 (12)
Total proteins gm./100 ml. plasma	6.6 (5)	4.6 (6)	4.3† 5.8 (6) (21)	3.0 4.1 (8) (17)	1.7 (12)
Albumin gm./100 ml. plasma	2.2 (5)	—	2.5 (6)	1.5 (8)	0.74 (12)
Globulin gm./100 ml. plasma	4.4 (5)	—	1.7 (6)	1.58 (8)	0.95 (12)
Blood urea nitrogen mg./100 ml. plasma	7.6 (9)	—	8.6 (15)	8.0 (7)	8.8 (12)
Protein-bound iodine μg/100 ml. plasma	13 (12)	4.5 (1)	5.2 (10)	2.3 (17)	— —
Red blood cells millions	2.04 (5)	2.54 (6)	1.66 (9)	1.64 (10)	2.02 (10)
Hemoglobin gm./100 ml. blood	8.6 (5)	10.8 (9)	12.1 (5)	11.8 (8)	9.8 (9)
Packed cell volume volume percent	49 (5)	57 (10)	60 (9)	65.7 (10)	43.6 (10)

* Figures in parentheses = numbers of fish studied.

† Only in these fish out of the total number of migrating spring-run and spawning fall-run salmon tested for total proteins, were albumin and globulin fractions determined.

cessible to salmon between April or May and the first fall rains, due to low stream flow, it is certain that these salmon have been in the upper stream section near the spawning beds for at least 4 months. The second group consisted of a fall run, most of which leave the sea from August through October and spawn in the period October through December. Their gonads are in a much more advanced stage of maturation at the beginning of the migration into fresh water than are those of spring-run fish. A majority of the fish in this run are in fresh water no longer than 2 to 3 months before spawning. A limited study was made of another fall run which spawn in the American river after only 1 to 2 months in fresh water.

Data on the lengths of time spent by the two runs of salmon in the river, stated above and shown in Tables 1 and 2, were furnished by fisheries' biologists who have studied salmon runs in the Sacramento River system over a period of years. For more details

TABLE 2. SEX DIFFERENCES IN VARIOUS BLOOD CONSTITUENTS IN SPAWNING SALMON OF FALL AND SPRING RUNS

	Sex	Fall-run	Spring-run
17-OHCS μg/100 ml. plasma	♂ ♀	31 79	108 100
Glucose mg./100 ml. plasma	♂ ♀	220 173	138 98
Cholesterol mg./100 ml. plasma	♂ ♀	436 303	157 83
Total proteins gm./100 ml. plasma	♂ ♀	4.0 4.2	2.0 1.1
Number of red blood cells—millions	♂ ♀	1.75 1.54	2.31 1.74
Hemoglobin gm. percent	♂ ♀	13.4 11	11.5 8.1
Packed cell volume percent	♂ ♀	70 61	49.8 37.4

about the runs of king salmon in this river the reader is referred to a paper by Hallock, Fry and La Faunce (8).[4]

Methods.—Individual fish were studied in as close to their natural state as possible. Sea fish were allowed a brief period of recovery in aerated tanks before study. River fish were caught in large traps or seins. In some instances, river fish were held overnight in "live cars" before study. Results of various tests on these fish were the same as those obtained on freshly caught salmon.

Blood was obtained by cardiac puncture as described previously (7). In the latter part of the study mepesulphate (Hoffman-LaRoche) 250 mg./ml. water was substituted for heparin as anticoagulant. After removing aliquots for erythrocyte counts, for hemoglobin and packed cell volume determinations, the blood was centrifuged and the plasma separated and stored on ice. Whenever assay was delayed beyond 24 hours, the plasma was frozen until required for analysis.

Because of the difficulty in securing adequate amounts of blood from salmon taken in the sea, due to the shock-like state of the fish caught by hook and line, it was necessary to pool the blood for various chemical determinations. Hematological and electrophoretic procedures, however, were done on individual sea fish.

Routine clinical methods were employed for the determination of glucose, urea nitrogen, cholesterol and cholesterol esters, total proteins, protein-bound iodine, erythrocyte counts, hemoglobin and packed cell volume; 17-hydroxycorticosteroids in plasma were measured by the method of Peterson and Wyngaarden (9) and Silber and Porter (10). Paper strip plasma electrophoresis (11) analyses were done with Whatman number 1 strips and the B_3 cam of the earliest Spinco integrating photoelectric scanner. Sodium and potassium were measured by flame photometry.

Urine was tested for presence of glucose with Testape® (Lilly) which was wet with urine obtained by aspiration of the genital pore or expressed by pressure over the lower abdomen.

[4] We are indebted particularly to Mr. Richard J. Hallock for checking and adding details to the above statement concerning lengths of time the salmon of various runs had spent in fresh water prior to spawning.

Radio-iodine uptake by the thyroid gland was determined at the end of 24 hours after intramuscular injection of 12.5 microcuries of radioactive iodine. The floor of the mouth containing the thyroid tissue was excised and used for measurement and for comparison with a standard. A specimen of muscle was secured for tissue background measurement.

RESULTS

Wherever possible, separate determinations on each animal were done and the results averaged. In some instances, blood samples were pooled in order to obtain adequate quantities of plasma for study. A characteristic of these observations is the wide range of values noted both within a group studied at one time and place and between individual values obtained at different times, but at the same collecting point. The differences between spring-run and fall-run spawning salmon were so consistent that they are presented separately. The results of the various determinations are summarized in Table 1.

17 Hydroxycorticosteroids.—Estimation of corticoids in spawning spring-run salmon (not heretofore reported) revealed a concentration of over 100 μg/100 ml. plasma. These fish had been in fresh water approximately twice as long as the salmon previously studied. Data on the earlier published findings are added to the table for comparison.

Glucose.—One of the most striking changes in the blood of salmon during the spawning migration is the increase in glucose content. Plasma glucose of salmon taken at a point on their migration 285 miles up the river (spring-run) was found to be twice that of the sea salmon—210 mg./100 ml. plasma (with a range of 121 to 286 mg.) as compared to 99 mg./100 ml. plasma in the sea salmon.[5] At this stage the gonads were, with few exceptions, in their early stages of development. By the time full sexual maturity had been reached, some 4 to 5 months later, the glucose content had fallen to a considerably lower level; in the female it was essentially the same as in the sea salmon. An occasional male exhibited well-marked hypoglycemia as low as 35 mg./100 ml. plasma. The fall-run salmon taken 125 miles upstream on their spawning migration, showed a degree of hyperglycemia similar to that found in spring-run males. However, unlike the spring-run salmon, the high concentration of blood glucose in these fish remained essentially unaltered until the time of spawning. The urine contained no glucose except in a very few instances where a trace of glucose was detected only in spawning salmon.

Sodium.—A reduction in the concentration of sodium was found in the plasma of spawning salmon—more marked in the fall-run than in the

[5] It is quite possible that the normal blood glucose of the sea salmon would be found to be lower than this figure if blood could be obtained before the intense struggle of the fish being brought in by hook and line. Black (12, 13) found an appreciable rise in the levels of blood glucose in Kemloops trout and lake trout following 15 minutes of severe muscular exercise.

spring-run fish. This change occurred as the salmon matured. An occasional salmon taken 285 miles upstream showed no diminution in the sodium content of the blood plasma from the average in the sea and in the case of the fall-run fish the principal change occurred weeks to months after the fish had entered the river. In a separate study of immature Columbia River king salmon-grilse[6] (showing early testis maturation), taken 500 miles upstream, the plasma sodium concentration averaged 170 m. eq./L.

Potassium.—After the fish entered fresh water, the potassium content of the salmon's plasma decreased. In spawning fall-run fish the plasma potassium was one-third of the concentration in sea fish. In spring-run salmon, there was an early decrease of plasma potassium in the migrating fish to less than one-half the level in the sea fish. However, at the time of spawning the potassium was found to have returned to the original concentration observed in immature salmon.

Cholesterol.—The highest cholesterol values were found during migration at a stage when the gonads were still small. Individual determinations varied widely. The mean for the group of spring-run salmon was 745 mg./100 ml. plasma (754 mg. for males and 512 mg. for females). Maximum concentrations for males were 1470 mg. and for females 920 mg. Cholesterol esters were found to constitute approximately two-thirds of the total cholesterol. By the time of full sexual maturity and spawning of the spring-run group, the cholesterol concentrations had fallen to levels far below those present in sea salmon—157 mg./100 ml. plasma in males and 83 mg. in females (Table 2). On the other hand, the concentration of plasma cholesterol in the spawning fall-run fish showed only a moderate decrease from that found earlier during their migration up stream. The greater diminution in blood cholesterol of the spawning spring-run salmon could be due to the much longer period of starvation as compared with the fall-run fish.

Blood urea nitrogen.—This constituent of the blood plasma remains essentially constant during the entire sexual cycle.

Total protein, albumin and globulin.—Changes in concentration of blood protein are displayed in Table 1. The amount of protein in the blood plasma diminished during the spawning migration. The lowest level was found in salmon which had endured the longest period of starvation. This was approximately one-fourth the concentration present in immature salmon in the sea. It should be noted that the albumin-globulin ratio of 1:2 in the sea was reversed during migration, then at spawning changed again to an equal or higher proportion of globulin. The significance of these changes is unknown.

A considerable number of paper-strip electrophoretic determinations were made of the sera of salmon at different stages of the sexual cycle. No

[6] Young male—1 to 2 years of age.

satisfactory interpretation of the results is possible at this time since we have no information as to whether the protein peaks of the salmon serum represent the same components that we are accustomed to find in mammalian sera. If, during electrophoresis, the movement of the salmon proteins resembles that of the human, then our data indicate that both gamma and alpha globulins were in the low normal range compared to human standards. Individual fish showed wide variations in gamma globulins. In some instances, despite low total proteins and albumin levels, the gamma

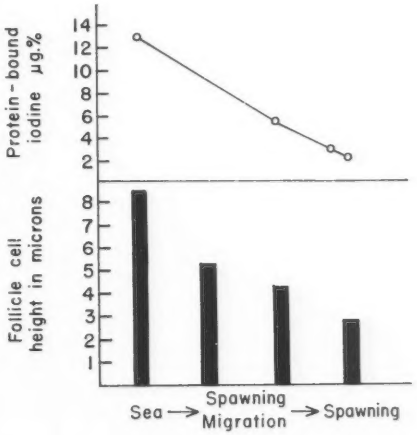

FIG. 1. Changes in plasma protein-bound iodine concentration and thyroid follicle cell height in salmon during spawning migration.

globulin concentration was as high in the spawning fish as in the sea fish. These relationships will be dealt with in a further communication (14).

Protein-bound iodine.—The plasma protein-bound iodine fell from a level of 13.3 µg/100 ml. plasma in the sea to 2.3 µg at the time of spawning (Table 1).[7] The evident diminution in the concentration of circulating thyroid hormone paralleled the decrease in heights of the thyroid follicle cells found to occur as the fish progressed on the spawning migration (6). This relationship is shown in Figure 1.

Uptake of I^{131}.—The 24 hours radioactive iodine uptake by the thyroid yielded values between 2% and 4% in the two groups of migrating and spawning salmon. Fontaine and Fontaine found 1% to 2% I^{131} uptake in 24 hours in both migrating and spawning Atlantic salmon (*S. salar*) (15).

Red blood cells, hemoglobin and packed cell volume.—Certain quite definite changes in the formed elements of the blood were detected during the several stages of the sexual cycle (Table 1). The decrease in number of red blood cells with increase in hemoglobin and packed cell volume of the

[7] We wish to express our very sincere appreciation to Dr. Harold Malkin, Malkin Clinical Laboratory, Palo Alto, for carrying out tests for protein-bound iodine on a considerable number of salmon plasma samples.

migrating spring-run fish as compared with the sea fish suggested a possible increase in the size of the red cells as well as some loss in numbers. At spawning, the blood picture tended to revert to that of the immature salmon in the sea. The spawning fall-run salmon exhibited changes similar to those of the migrating spring-run fish. With a view to obtaining some understanding of the changes in the salmon's red blood cell count, blood volume determinations were attempted, using radio-iodinated human serum albumin. Preliminary tests have been technically unsuccessful.

Sex differences.—In certain of the physiological changes we have studied, there was a marked difference between male and female salmon. When present, these differences were most accentuated at the time of spawning. Such data on spawning spring-run and fall-run fish are presented in Table 2. It will be noted that values were lower in females with two exceptions occurring in fall-run salmon, where corticoids in the females were much higher than in the males and the total proteins were essentially the same in both sexes. No significant differences between male and female salmon were detected in plasma electrolytes, protein-bound iodine or blood urea nitrogen levels.

Other characteristics of the spawning salmon.—The maturing salmon, particularly the male, develops striking morphological changes not encountered in immature fish. Overgrowth of the cartilage of the jaws, and a hump back (16), (Figs. 2, 3 and 4). These changes are accompanied by a considerable weight loss—most pronounced in salmon which have been in the river for many months. In some studies, as much as 30% loss of the muscle protein has been reported, along with most of the muscle fat (17). By the time of sexual maturity, fish usually are weak and react poorly to stress, e.g., violent exertion induced by handling. During migration, there is an increasing incidence of infection as indicated by the appearance of fungus growth on the skin. During residence in holding ponds at hatcheries, these infections have been observed to spread over the whole body within a few days.

No roentgenological evidence of demineralization indicative of osteoporosis was found in comparative X-ray studies of the vertebrae of salmon taken at several points on their upstream migration and at the spawning ground of fall-run fish. Bones of spawning spring-run salmon have not been examined.

DISCUSSION

A number of workers have reported observations on the changing concentrations of various blood constituents in salmon, both Pacific and Atlantic, during their fluvial spawning migration. However, with one exception (Greene's electrolyte studies (24)), these investigations do not include data on salmon taken in the sea, and hence the findings lack a base

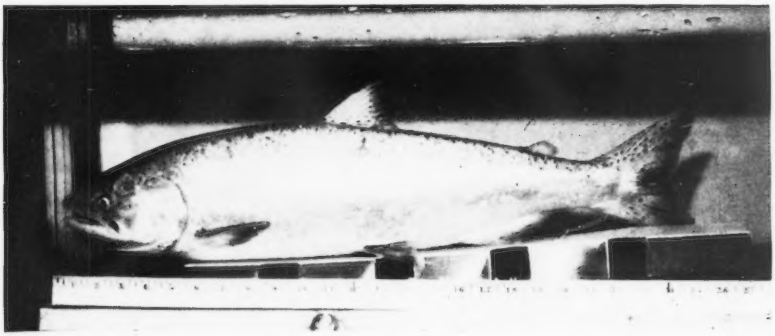

FIG. 2. Male king salmon 24 inches in length taken on spawning migration. Testes in early stages of development. Silvery, skin clean.

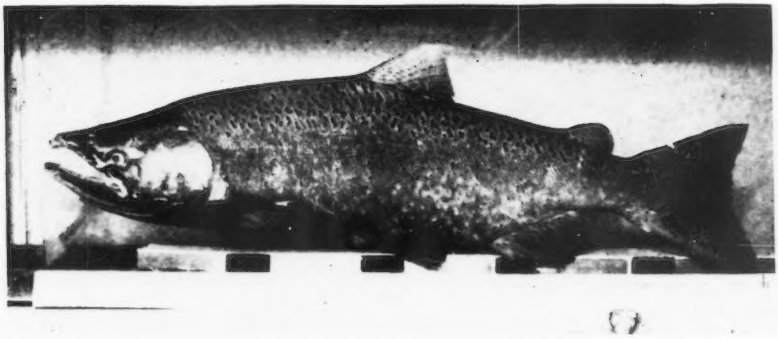

FIG. 3. Male king salmon 24 inches long taken on spawning ground. Jaws prolonged and hooked. Back shows a well marked hump. Skin dark, fungus infection beginning on eye and flank posteriorly.

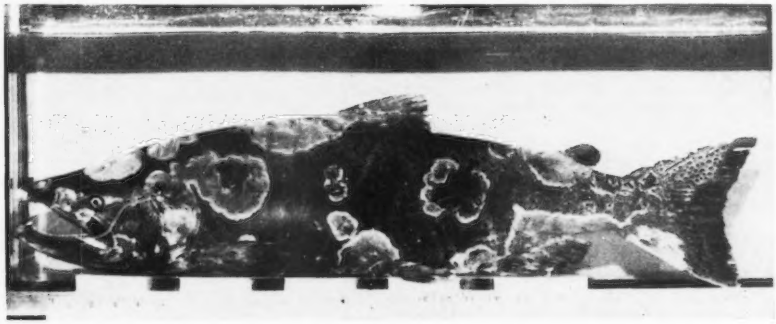

FIG. 4. Male king salmon 31 inches in length taken on spawning ground. Jaws prolonged and hooked, back has slight hump, skin dark and shows extensive patches of fungus infection.

line for comparison with subsequent changes which occur after the salmon enters the river. The available data are displayed in Table 3.

It will be noted that these observations differ in a number of respects from our findings (Table 1). Even in the same genus of salmon, though not the same species, Idler *et al.* (18) found much higher corticoid concentrations in spawning sockeye than we did in spawning chinook salmon. However, both species of Pacific salmon showed a progressive rise in the level of 17-OHCS during migration, whereas in the spawning Atlantic salmon, Fontaine and Hatey (19) found a decrease from earlier higher values.

Whether the concentrations of blood glucose reported by Fontaine and Hatey (20) in S. salar represent hyperglycemia is not apparent in the absence of data on this fish in the sea. The concentrations of blood glucose in our Pacific salmon during the spawning migrations was almost twice that of the Atlantic fish, although the 17-OHCS levels were about the same in both. Another striking difference between these two salmons is the degree of glycogen storage in the liver. Fontaine and Hatey (20) found 4 to 7 times more glycogen in the liver of their Atlantic salmon than did Greene in the Pacific species (Table 3).

TABLE 3. SUMMARY OF DATA REPORTED BY OTHER WORKERS ON BLOOD CONSTITUENTS AND LIVER GLYCOGEN OF MIGRATING AND SPAWNING SALMON

	Kind of salmon	Sea immature	During spawning migration	Sex	At spawning
17-Hydroxycorticosteroids μg/100 ml. plasma					
Idler, Ronald and Schmidt (18)	Pacific (O. nerka)	—	33	♂	154
			78	♀	231
Fontaine and Hatey (19)	Atlantic (S. salar)	—	52	♂	32
			45	♀	31
Glucose mg./100 ml. blood					
Fontaine and Hatey (20)	Atlantic	—	123	♂	84
			96	♀	74
Liver Glycogen mg./gm. liver					
Greene (21)	Pacific (O. tshawytscha)	3.7 ♂ ♀	—	♂	0.4
				♀	0.4
Fontaine and Hatey (20)	Atlantic	—	27	♂	24
			18	♀	0.5
Electrolytes—lowering of freezing point					
Greene (24)	Pacific (O. tshawytscha)	−0.762° C	−0.737° C		−0.612° C
Fontaine and Koch (25)	Atlantic	—	−0.75° C		−0.66° C
Cl. meq./L serum	Atlantic	—	134		129
Cholesterol mg./100 ml. plasma					
Idler and Tsuyuki (29)	Pacific (O nerka)	—	261	♂	131
			215	♀	77
Protein-bound Iodine μg/100 ml. plasma					
Fontaine *et al.* (30)	Atlantic	—	29.7		14

The very small amount of glycogen in the liver (and muscle) of the Pacific salmon even in the sea (21) suggest that the hyperglycemia developing during sexual maturation in the river is due predominantly to gluconeogenesis resulting from the action of adrenal corticoids on muscle and fat depots. Our finding of hypertrophy of the islands of Langerhans with marked increase in the numbers of beta cells in the sexually mature salmon is in keeping with an increased requirement for insulin occasioned by hyperglycemia (22, 23).

Decrease in blood electrolyte concentration in anadromous fishes migrating from salt water to fresh water is well recognized and is reviewed comprehensively by Fontaine and Koch (25). Greene's (24) finding of a 20 per cent rise in the freezing point of serum from sea to spawning ground (Table 3) corresponds fairly closely to the fall we found in plasma sodium concentrations. Decrease in blood electrolytes of the Pacific salmon would appear to be greater than that found by Fontaine and Koch in *Salmo salar*, but, as they point out, individual variations were marked and certain of their fish showed essentially no lowering of blood electrolyte levels even after reaching the spawning ground. Our determinations of sodium concentration in migrating and spawning salmon likewise showed a wide individual range.

The effect of adrenal corticoids on osmoregulation in fish is unsettled. Spaulding (cited by Chester Jones (26)) showed that the injection of cortisone in brown trout caused a decrease in concentration of both sodium and potassium in the blood, but that ACTH produced an increase in sodium and a decrease in potassium. Chester Jones, Phillips and Holmes (27) presented experimental evidence to show that adrenal corticoids, hydrocortisone and desoxycorticosterone, increased the sodium output from rainbow trout after an injected load of NaCl, and that this enhanced secretion occurred through the gills. What relationship their findings may bear to the normal physiology of a trout is uncertain. We know of no experiments in fish on the effects of aldosterone on sodium excretion, but the recent findings by Phillips, Holmes and Bondy (28) of aldosterone in the blood of spawning Pacific salmon, suggests the possibility that this hormone may act to prevent a marked drop in plasma sodium despite starvation and prolonged sojourn in fresh water.

We have no information as to whether the lowered concentration of potassium in the plasma, which we observed constantly in migrating and fall-run spawning fish, is due to high corticoid levels or to starvation or both. The relatively high plasma potassium in the spawning spring-run fish is inconsistent with other observations, but it may be due to release of intra-cellular potassium accompanying the rapid tissue deterioration of these spawning and spent salmon.

Fontaine and co-workers (30) found a decrease of thyroid follicle cell height and of plasma protein-bound iodine in Atlantic salmon during

TABLE 4. COMPARISON OF BIOLOGICAL, BIOCHEMICAL AND ANATOMICAL CHANGES IN CUSHING'S SYNDROME IN HUMAN BEINGS AND IN SPAWNING SALMON

Changes	Cushing's syndrome	Spawning salmon
A. *General*		
Weight	Gain or no change	Loss (starvation)
Muscle tissue	Wasted	Wasted
Weakness	Present	Present
Osteoporosis	Present	Absent (?)
Increased susceptibility to infection	Present	Present
Increased sensitivity to stress	Present	Present
Blood pressure	Hypertension	—
B. *Blood*		
17-OHCS conc.	High	High
Glucose	Hyperglycemia	Hyperglycemia
Sodium	Normal or increased	Decreased
Potassium	Normal or decreased	Decreased
Total serum protein	Decreased	Decreased
Gamma globulin	Decreased	Variable
Cholesterol conc.	Increased	Decreased
Blood urea nitrogen	Normal	Normal
Protein-bound iodine	Normal or decreased	Decreased
Polycythemia	Present	Absent
C. *Histology*		
Adrenal	Hyperplasia	Hyperplasia
Pituitary	Hyaline degeneration of basophils	Degeneration of both basophils and acidophils
Kidney	Nephrosclerosis	Nephrosclerosis
Pancreas	Islet hypertrophy variable	Islet hypertrophy constant
Lymphoid tissue	Depletion	Depletion
Liver	Degeneration variable	Degeneration constant
Cardiovascular system	Arteriosclerosis	Degenerative changes beginning arteriosclerosis

migration to the spawning grounds analogous to, but less pronounced than, our findings in Pacific salmon. The marked reduction in protein-bound iodine occurring in our spawning fish correlates well with the histological changes, atrophy and degeneration in the thyroid gland.

It seems evident from the above data, that alterations in the blood of the Atlantic salmon, in so far as they have been studied, exhibit only a partial resemblance to those occurring in the Pacific genus (Tables 1 and 3). The nature of the changes taking place in the migrating and spawning Pacific salmon would appear to be consistent with the effects of hyperactivity of the adrenal cortical tissue. Both biochemical and anatomical changes are similar in many respects to those observed in experimental hyperadrenocorticism in animals and in the spontaneous form occurring in humans (Cushing's syndrome). In Table 4 is a comparison of the characteristic changes in Cushing's syndrome and the spawning salmon.

Differences between these two examples of hyperadrenocorticism are to be expected in view of the extended starvation of the salmon and the depletion of various body elements to supply energy for migration and the for-

mation of gonadal tissue. This makes it all the more remarkable that the correspondence between Cushing's syndrome and the spawning salmon is so close. Certain features prominent in the former and not found in the latter, e.g. osteoporosis, are possibly due to the relatively brief period of exposure to high concentrations of corticosteroids. The drop in the concentration of plasma cholesterol late in the course of sexual maturation in the salmon could be a result of starvation, prodound somatic depletion and liver degeneration. Polycythemia, a common finding in Cushing's syndrome, is not present in salmon at the time when adrenal corticoids are elevated. Starvation and alterations in hydration during months spent in fresh water may produce changes which would prevent the development of polycythemia. Histological changes occurring in the tissues of patients with Cushing's syndrome have their counterparts in the spawning salmon. With the exception of the cardiovascular system (31) these are more pronounced in the salmon (6).

The primary event leading to hyperadrenocorticism in the Pacific salmon is most likely to be found in increased stimulation of the pituitary gland via the hypothalamic centers. The histological changes in the pituitary would support this view. Whether one is dealing with an intrinsic stimulation to increase both ACTH and gonadotropin secretion or whether the adrenal cortical hyperactivity is secondary to and dependent on gonad development as suggested in a previous study (5, 32) and what role the stresses of migration (5) may play, are questions which cannot be decided upon at this time.[8]

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SEX DIFFERENCE IN THE RATE OF DISAPPEARANCE OF CORTICOSTERONE-4-C^{14} FROM PLASMA OF INTACT RATS: FURTHER EVIDENCE FOR THE INFLUENCE OF HEPATIC Δ^4-STEROID HYDROGENASE ACTIVITY ON ADRENAL CORTICAL FUNCTION[1]

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ABSTRACT

The half-time for the disappearance of corticosterone from plasma in female rats is much shorter than that in males. This large sex difference in steroid metabolism *in vivo* corresponds to the *in vitro* sex difference in corticosteroid inactivation by hepatic Δ^4-steroid hydrogenases, but is opposite to the sex difference in cortisone metabolism by hepatic C20-carbonyl hydrogenases. From calculations based on these data we conclude that the half-life is ordinarily independent of hepatic blood flow. The steady-state rate of corticosterone secretion was estimated in both sexes. Under the conditions of these experiments the female adrenal cortex appears to secrete corticosterone *in vivo* at a steady-state rate more than twice as great as that of male adrenals. The virtual volume of distribution and the half-life of corticosterone both appear to be independent of the dose of corticosterone administered, over a four-fold physiological concentration range.

THE discovery that livers of adult female rats have more than twice the capacity of those from males to saturate ring A of adrenal cortical hormones *in vitro* has been offered as an explanation for the larger adrenal cortex of the female rat and as new evidence for the negative feedback control of adrenal cortical secretion (1, 2). But recently it has been found that livers of males reduce the C20 carbonyl group of cortisone faster than do those from females, *in vitro* (3, 4). Since either of these reactions is sufficient to cause physiological inactivation of adrenal cortical hormones, we have undertaken *in vivo* studies of the disappearance of corticosterone from rat plasma, to see which of these hepatic reactions is physiologically dominant in this species.

METHODS

Male and female Sprague-Dawley rats offered Purina chow and water *ad lib.* were used in all experiments.

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Determination of the half-life of corticosterone in rat plasma

The animals were anesthetized with 5 mg./100 gm. B. W. of pentobarbital intraperitoneally. 0.25 ml. of alcohol-saline containing a total of 50 μg of corticosterone including 0.2 μc of corticosterone-4-C^{14} (150,000 counts per minute in our system) was injected intravenously at zero time. Five or six 0.3 ml. venous blood samples were withdrawn at 5 to 15 minute intervals from another site. After centrifugation of the blood, 0.1 ml. plasma samples were plated on lens paper, dried, and counted at a sample thickness of 2.3 mg./cm.^{2} for 2,000 counts in a helium-butane quenched, end-window, gas flow counter operating in the Geiger region. The background noise was such that the standard deviations of the counts on the initial samples at 10 minutes averaged 2.8%, while that of the least active 60-minute sample ever observed was 22.6%. The least squares line relating the log cpm/0.1 ml. plasma to time elapsed was used to calculate the half-life of the corticosterone. In spite of the often large standard deviations of the later samples, all the raw data from each animal fell on or close to the least squares line.

To determine whether the radioactivity in the plasma at the times observed following injection of labelled corticosterone was indeed predominately from corticosterone-4-C^{14}, and not from recirculated metabolites of the steroid, we extracted samples of plasma sequentially with iso-octane (2, 2, 4 trimethyl pentane), 0.1N NaOH and $CHCl_3$. The chloroform fraction was then chromatographed in a toluene: 70% methanol system, using pure corticosterone as a reference standard.

Determination of plasma corticosterone concentration

Blood samples were obtained by decapitation from unanesthetized rats. Collection was complete within 10 seconds of seizure of the animals. The rats were kept in shielded cages, and precautions were taken against disturbing them before they were to be killed. The corticosterone concentration was measured by a method involving fluorescence in sulfuric acid (5, 6) as modified by Guillemin *et al.* (7).

Data presented as means are accompanied by the standard errors of the means.

RESULTS AND DISCUSSION

Identification of the plasma radioactivity as corticosterone-4-C^{14}

A small amount of labelled corticosterone was added to rat plasma *in vitro*, and then incubated with shaking at 37° C for 20 minutes, to simulate the conditions of the *in vivo* experiments, but without the possibility of hepatic metabolism of the steroid. Subsequent extraction with iso-octane, 0.1N NaOH and chloroform revealed that 93% of the recovered counts were in the chloroform fraction.

When plasma samples obtained during an *in vivo* half-life study were similarly extracted, 86% of the total counts recovered were in the chloroform fraction. This fraction was then chromatographed, and the distribution of counts was determined before and after elution with absolute ethyl alcohol. In samples obtained 20 minutes after injection of labelled corticosterone at least 66% of the total radioactivity eluted was in the region of the corticosterone standard in both sexes. In samples obtained 40 minutes after injection 81% of the total radioactivity eluted was in the corticosterone region in females, and 91% in males. In the early samples from males the non-corticosterone radioactivity was localized in two zones more polar

than corticosterone, whereas in those from females it was localized in three more polar zones.

From these studies we concluded that although in both sexes plasma radioactivity during the first 40 minutes after intravenous injection of corticosterone-4-C^{14} represents predominantly the unaltered steroid, the concentration of recirculated metabolites is sufficiently great that the half-time of the disappearance of radioactivity from unextracted plasma can be used only as an approximate measure of the half-life of corticosterone. Nevertheless, since there was no sex difference in the fraction of radioactivity which represented metabolites, the method was adequate to test for sex differences in corticosterone half-life.

Sex difference in biological half-life of corticosterone

The results are shown in Figures 1 and 2. The disappearance of corticosterone followed first-order kinetics exactly in both sexes during the interval studied. The half-life of corticosterone in the plasma of male rats was 22.2 ± 1.55 minutes. A similar half-life in male rats has been reported by Ulrich and Long (8). In contrast, in female rats the half-life was only 12.7 ± 0.83 minutes ($P < 0.001$). In man the half-life of corticosterone depends upon the dose of steroid administered to test it (9). However, as shown in Figures 2 and 3, the half-life in these studies was independent of the initial (10 minute) concentration over a four-fold concentration range, and the sex difference was still present in males and females with the same initial concentrations of labelled corticosterone.

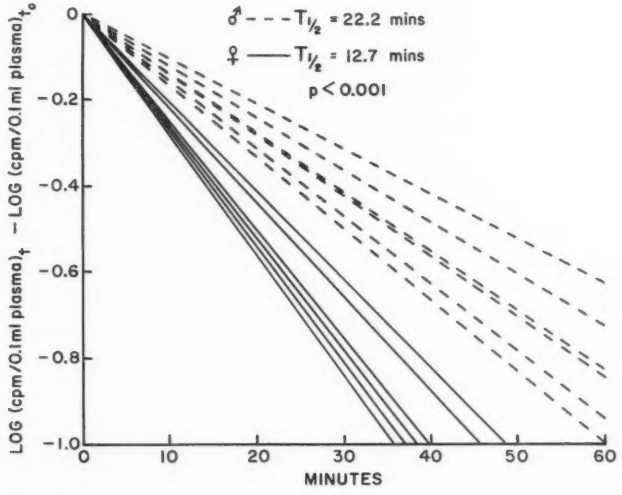

FIG. 1. Sex difference in the half-life of corticosterone in rats. Each line represents the least-squares line fitting five or six experimental points per animal.

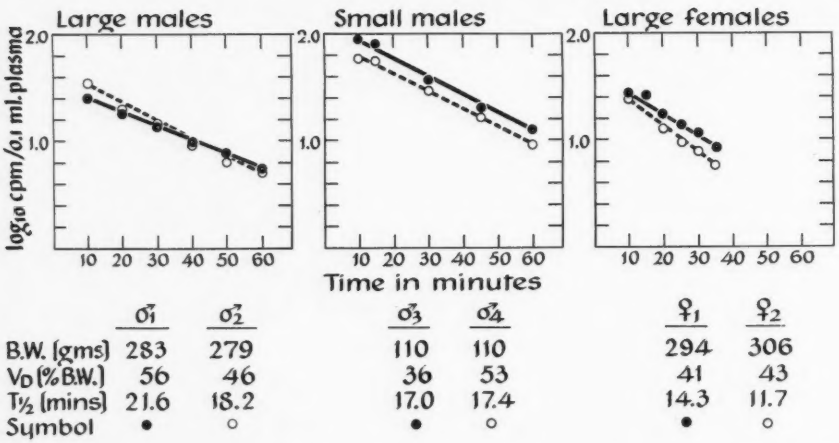
Large males
Small males
Large females
log10 cpm/0.1 ml. plasma
2.0
1.0
10 20 30 40 50 60
Time in minutes
♂1 ♂2 ♂3 ♂4 ♀1 ♀2
B.W. (gms) 283 279 110 110 294 306
VD (% B.W.) 56 46 36 53 41 43
T½ (mins) 21.6 18.2 17.0 17.4 14.3 11.7
Symbol • ○ • ○ • ○

FIG. 2. First order kinetics of the disappearance of radioactivity from plasma in large male, small male and female rats following intravenous injection of corticosterone -4-C^{14}. The sex difference in half-life is present in males and females of the same weight and same initial (10 minute) concentration of radioactivity.

Virtual volume of distribution of corticosterone

The virtual volume of distribution (i.e. the single-compartmental volume of distribution at plasma concentration) of corticosterone-4-C^{14} during the hour after injection of the labelled steroid was obtained from extrapolation of the disappearance curves to zero time, and from the known number of counts per minute injected. We did not make any correction for urinary losses of the injected material, and so the volumes of distribution are overestimated, but the error is probably small since urinary excretion of radioactive substances following administration of labelled corticosteroids is low in the rat even after large doses of the hormones (10). The results are shown in Table 1 and Figures 2 and 4.

In five male rats weighing between 280 and 350 grams, the volume was $45 \pm 3.2\%$ of total body volume (weight). In six males weighing between 100 and 132 grams it was $46 \pm 3.5\%$. In two females of approximately 300 grams the volume was 42% of body weight. The virtual volume of distribu-

TABLE 1. RELATIONSHIPS BETWEEN BODY WEIGHT AND THE LIVER WEIGHT, VIRTUAL VOLUME OF DISTRIBUTION OF CORTICOSTERONE, AND THE HALF-LIFE OF CORTICOSTERONE, IN MALE RATS

Body weight range (grams)	No. of animals	Liver weight (% B. W.)	Virtual volume of corticosterone distribution (% B. W.)	Half-life of corticosterone (minutes)
100–132	6	4.38 ± 0.06	46 ± 3.5	18.2 ± 1.0
280–350	5	4.00 ± 0.04	45 ± 3.2	21.2 ± 1.1

TABLE 2. PLASMA CORTICOSTERONE CONCENTRATIONS IN MALE AND FEMALE RATS

The groups of animals bled in the afternoon have been arbitrarily subdivided into those which struggled or squealed during handling, and those which were quiet. A greater responsiveness of the female to environmental disturbances is indicated. A diurnal increase in concentration in the afternoon is evident in the males.

Sex	Plasma corticosterone conc. (μg/100 ml.)			
	8–9 A.M.	2–3 P.M.		
	All animals	All animals	Quiet animals	Struggling animals
Males	8.0 ± 0.58 (11)	13.7 ± 0.6 (45)	12.2 ± 0.16 (26)	15.8 ± 0.4 (19)
Females	15.0 ± 2.09 (8)	25.3 ± 3.0 (16)	16.9 ± 1.72 (6)	30.2 ± 1.32 (10)

tion thus appears to be proportional to body weight, and is approximately the same fraction of total body weight in both sexes.

In adult humans Peterson and Pierce found that the volume of distribution of corticosterone depended on the dose of steroid given: for trace doses it was apparently the same fraction of body weight as we report here for the rat (9). As shown in Figure 4, over a four-fold concentration range the volume of distribution is independent of the initial concentration (dose) in the present studies.

The dependence of both distribution volume and half-life of corticosterone (and cortisol) in man upon the dose of the steroid administered has been observed when tracer doses were compared to pharmacological doses. The four-fold initial concentration range of labeled corticosterone in our experiments in the rat was in most cases associated with total plasma corticosterone concentrations less than the maximal level which can be produced physiologically by the rat. For example, a dose of corticosterone of

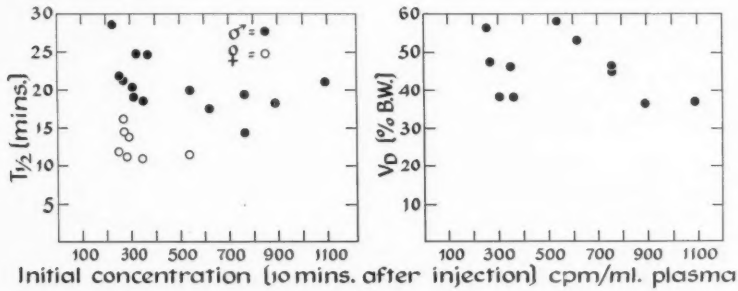

FIG. 3. (Left) Independence of the half-life of corticosterone from the dose of corticosterone administered over a four-fold physiological dose range. The sex difference in half-life is present in males and females receiving the same dose of corticosterone (i.e.—with the same initial plasma concentration of corticosterone-4-C^{14}).

FIG. 4. (Right) Independence of the virtual volume of distribution of corticosterone in male rats from the dose administered (as indicated by the initial concentration of radioactivity in plasma) over a four-fold range.

35 μg/100 gm. B. W. I.V. produced after 15 minutes a level of 54 ± 1.73 μg/100 ml. in eight rats, whereas restraint produced levels which approach 70 μg/100 ml. after fifteen minutes. The corticosterone-4-C^{14}, used in the present experiments was administered in a total dose of corticosterone of 50 μg, in animals ranging from 100 to 350 grams in weight. The highest dose used, therefore, was 50 μg/100 gm. B. W., which, as can be seen from the results with the 35 μg/100 gm. B. W. dose, probably produced plasma levels close to the physiological maximum in this species. Thus the doses used in this study were neither tracer, nor pharmacological doses. Consequently our failure to find a dose-dependence of distribution volume or half-life over the four-fold physiological concentration range does not necessarily indicate a species difference in the handling of corticosterone by man and the rat.

According to a single experiment reported by Slaunwhite and Sandberg (11), rat blood contains one or more corticosteroid-binding proteins which, in their system, bind corticosterone to about the same extent (75%) that it is bound in human plasma (72–78%) by proteins other than albumin. However, at present there is no detailed information concerning the binding capacity of albumin or of a specific corticosteroid-binding globulin in rat plasma. Our data showing that in the rat a *physiological* dose of corticosterone has about the same volume of distribution as does a *tracer* dose in man, may indicate a higher capacity of the binding proteins in the rat compared to man.

It seems to us that the dose-dependence of the half-life of corticosterone in man is difficult to explain on the basis of protein binding, since tracer doses which are nearly 100% bound have a shorter half-life than have pharmacological doses which are only 70–80% bound. However, the higher concentration of unbound corticosterone following pharmacological doses may prolong the half-life by depressing the activity of hepatic Δ^4-steroid hydrogenases, as we have shown large doses of ACTH or cortisone to do in the rat (2).

Effect of body weight and liver size on the half-life of corticosterone

The concentration of Δ^4-steroid hydrogenase activity in rat livers remains relatively constant as rats grow from 100 to 400 grams (2, and unpublished observations). The total enzymatic activity therefore increases almost linearly with body weight, because the liver remains a nearly constant fraction of total B. W. (Table 1). The capacity of an animal to inactivate adrenal cortical hormones by reduction of ring A in the liver is thus proportional to the size of the animal. However, in spite of this relationship, if the enzymatic process determines the rate of disappearance of corticosterone from plasma, the half-life of corticosterone would be expected to be nearly the same in large and small animals, since the virtual volume of distribution of the steroid is also proportional to body weight.[4]

To test this conclusion, the half-life was compared in the two groups of male rats, of very different body weights, described above. The results are shown in Table 1 and Figure 2. The half-life was slightly longer in the larger group, but the difference was not statistically significant ($P > 0.05$).

Plasma corticosterone concentration

The fluorescence method used to determine the plasma concentration of corticosterone cannot give absolute levels with certainty, since there is no way to determine with confidence how much of the fluorescence in a given sample comes from a non-corticosterone blank. The residual fluorescence observed in adrenalectomized or hypophysectomized animals does not necessarily represent the blank fluorescence in normal animals. We have therefore subtracted only the reagent blank from the fluorescence of the biological samples in calculating the concentration of corticosterone.

Blood samples obtained at approximately 8:00 A.M. from undisturbed normal adult male rats had corticosterone concentrations averaging 8.0 ± 0.58 μg/100 ml. plasma. The levels observed in females were much more variable, and over a period of one year have consistently averaged 5 to 7 μg/100 ml. plasma higher than those of males. The females appear to be more easily disturbed than do the males, and occasionally we have seen

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[4] The rate of removal of corticosterone from plasma followed first-order kinetics:

$$-\frac{dB}{dt} = k\left(\frac{B}{V}\right) \tag{1}$$

where B = amount of corticosterone in the virtual volume of distribution
V = virtual volume of distribution of corticosterone at plasma concentration
By integration of this equation, and solution for $t = T_{1/2}$, it can be shown that

$$T_{1/2} = \frac{0.7V}{k} \tag{2}$$

If an enzymatic reaction such as ring A reduction determines the rate of removal, then k will be proportional to the amount of active enzyme, whatever the order of kinetics of the enzymatic reaction itself. As shown above, the amount of active enzyme is itself proportional to body weight, and so

$$k = k'(\text{B.W.}) \quad \text{and} \quad T_{1/2} = \frac{0.7V}{k'(\text{B.W.})} \tag{3}$$

But we have shown that V/(B.W.) is nearly constant for animals in the size range studied, and so $T_{1/2}$ will be independent of the size of the animals.

The rate of adrenal cortical secretion of corticosterone necessary to maintain a steady-state plasma concentration of corticosterone in such a system must equal the rate of removal, and from (1) and (2) it is given by:

$$\frac{dB}{dt} = \frac{0.7V}{T_{1/2}}c \tag{4}$$

where c is the steady-state plasma concentration of corticosterone.

values as high as 45 μg/100 ml. plasma in samples from supposedly quiet, undisturbed females. Consequently, it is not certain that our "control" values of 8 μg/100 ml. in the males and 13 to 15 μg/100 ml. in the females really indicate a sex difference in the true "resting" corticosterone levels in this species. Rather, they may represent a sex difference in the responsiveness of males and females to environmental stimuli which we could not adequately control.

The levels obtained in two representative experiments, in which males and females were killed alternately between 8:00 and 9:00 A.M., or between 2:00 and 3:00 P.M. are shown in Table 2. A diurnal increase in levels in the afternoon can be seen in the males.

Our findings of a greater rate of inactivation of corticosterone by ring A reduction (1), and a shorter half-life for corticosterone in the plasma of females, do not imply that the true plasma corticosterone concentration in undisturbed females should be lower than that observed in males. If the negative feedback control of adrenal cortical secretion matches the rate of corticosterone secretion to the rate of ring A reduction of corticosterone in liver, as we have proposed (2), then the resulting steady-state level of corticosterone in plasma would be determined by the set point of the controller, and would be only slightly affected by variations in turnover rate. Even if the load-error of the controller were substantial, the level in the female could be maintained higher than that of the male, at all rates of turnover, by a higher set point in the female.

Steady-state secretion rate of corticosterone

From the volume of distribution, the half-life, and the plasma concentration of corticosterone, the secretion rate of corticosterone necessary to hold the plasma concentration at a steady level can be determined (equation 4). From the values obtained in this study (using the A.M. plasma corticosterone concentrations) the average steady-state secretion rates are 0.114 μg/min.·100 gm. B. W. in males, and 0.370 μg/min.·100 gm. B. W. in females. The adrenals of the males in this study weighed 15.3 ±0.05 mg./100 gm. B. W. and those of females weighed 26.4 ±0.3 mg./100 gm. B. W. Therefore the steady-state secretion rates *per milligram of adrenal* (not corrected for the slight amount of medullary tissue) were 0.0076 μg/min. in the male, and 0.014 μg/min. in the female. According to these calculations the female adrenal *in vivo* is much more active than the male adrenal, contrary to the view of McKerns (12).

The secretion rates we have arrived at from these calculations are only about one-fifth as great as those estimated by studies involving collection of adrenal venous blood (13–15), as would be expected, since our figures concern the steady-state secretion rates in quiet animals, whereas the direct measurements require some surgical disturbance of the animals, and probably indicate nearly maximal secretion rates.

The method we have used to arrive at the steady-state secretion rate is not as direct as the method used by Peterson in man, which involved the study of the change in specific activity of cortisol-4-C^{14} in plasma with time (16). This more elegant approach unfortunately requires numerous samples larger than can be obtained from individual rats.

Liver blood flow and steroid metabolism

The disappearance of corticosterone from plasma can be described by the usual Fick conservation equation. Since the liver is primarily responsible for the disappearance process (16–18):

$$-\frac{dB}{dt} = Q(c_i - c_0)$$

where Q is liver blood flow; c_i is the concentration of corticosterone in the incoming hepatic blood (hepatic artery or portal vein); and c_o is the concentration of corticosterone in blood leaving the liver in the hepatic veins.

If the liver extracted all the corticosterone in one pass through, so that c_o equalled zero, then $-(dB/dt) = Q\ c_i$ and under these special circumstances the sex difference in half-times could be accounted for entirely by a sex difference in hepatic blood flow, and the disappearance process would follow first order kinetics even if the rate of blood flow, rather than the amount of enzyme, were rate-limiting. Data from intact and perfused livers indicate that the liver does not extract all the corticosteroids reaching it in the blood or perfusion fluid (19, 20), and c_o is not zero, though occasionally it may be low if flow is low.

If c_o is not ordinarily zero, then it is very difficult to account for the sex difference in half-times of corticosterone in plasma on the basis of a sex difference in liver blood flow. The relation between the half-life and liver blood flow when c_o is not zero is given by:

$$-\frac{dB}{dt} = k\frac{B}{V} = Q(c_i - c_0) \qquad \text{From equation (2)} \quad k = \frac{0.7V}{T_{1/2}}$$

Therefore

$$T_{1/2} = \frac{0.7B}{Q(c_i - c_0)}$$

The sex difference in the amount of corticosterone in the virtual volume of distribution (B) is in the wrong direction to account for the sex difference in half-life, since according to our data females may ordinarily have more extra-adrenal corticosterone per 100 grams body weight than do males. Under the condition that $c_o \neq 0$, the sex difference in half-life could be entirely accounted for by a sex difference in hepatic blood flow (Q)

only if the extraction of steroid (c_i–c_o) were constant at all blood flows and were completely independent of the absolute values of the concentration of the hormone in the incoming plasma. But this restriction would require that at any given blood flow, the rate of disappearance of substrate be independent of c_i (i.e. the kinetics of the disappearance process would have to be zero order with respect to substrate concentration). The observed first-order kinetics of the disappearance excludes this possibility. It therefore appears more likely that changes in blood flow are associated with inverse changes in corticosteroid extraction, as has been suggested previously (19, 21), and that the half-life of corticosterone is thus independent of hepatic blood flow.

From these considerations we conclude that, with the obvious exception of instances in which hepatic blood flow approaches zero, and then has to be limiting, the amount of Δ^4-steroid hydrogenase activity in the liver determines the rate of disappearance of corticosterone from rat plasma. From studies on men with liver disease Peterson reached the similar conclusion that in humans the activity of hepatic Δ^4-steroid hydrogenases largely determines the rate of synthesis (release) of cortisol and corticosterone (22). Both these studies support our view that this hepatic enzyme system determines the secretion rate of adrenocorticotropin (ACTH) in undisturbed animals (2).

Summary of sex differences in adrenal cortical system of the rat

Figure 5 shows the various sex differences involving the adrenal cortical system of the rat. Our interpretation of these differences is that female rats have a greater amount of Δ^4-steroid hydrogenase activity in their livers, which leads to a greater rate of inactivation of adrenal cortical hormones by the liver of females. The sex difference in inactivation rates is less than the sex difference in the content of enzymes (compare slices and homogenates in the figure) partly because the kinetics of the two assays differ (2), but possibly also because the supply of the coenzyme triphosphopyridine nucleotide (TPNH) limits the activity of the enzyme to some degree. Female livers have a greater amount of glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase, and both of these enzymes catalyze reactions which generate TPNH (23). However, the sex difference in the TPNH generating system is smaller than that in the Δ^4-steroid hydrogenases in the homogenate assay and may limit the activity of the latter enzymes in females.

We believe that the present study indicates that the greater capacity for corticosteroid inactivation by liver in the female causes a sex difference in the half-life of corticosterone in intact rats. The half-life in the female is only about 50% of that in the male, while the rate of inactivation by ring A reduction (slices) is almost double that of the male. The sex difference in half-life represents a difference in the *in vivo* rate of corticosterone inactiva-

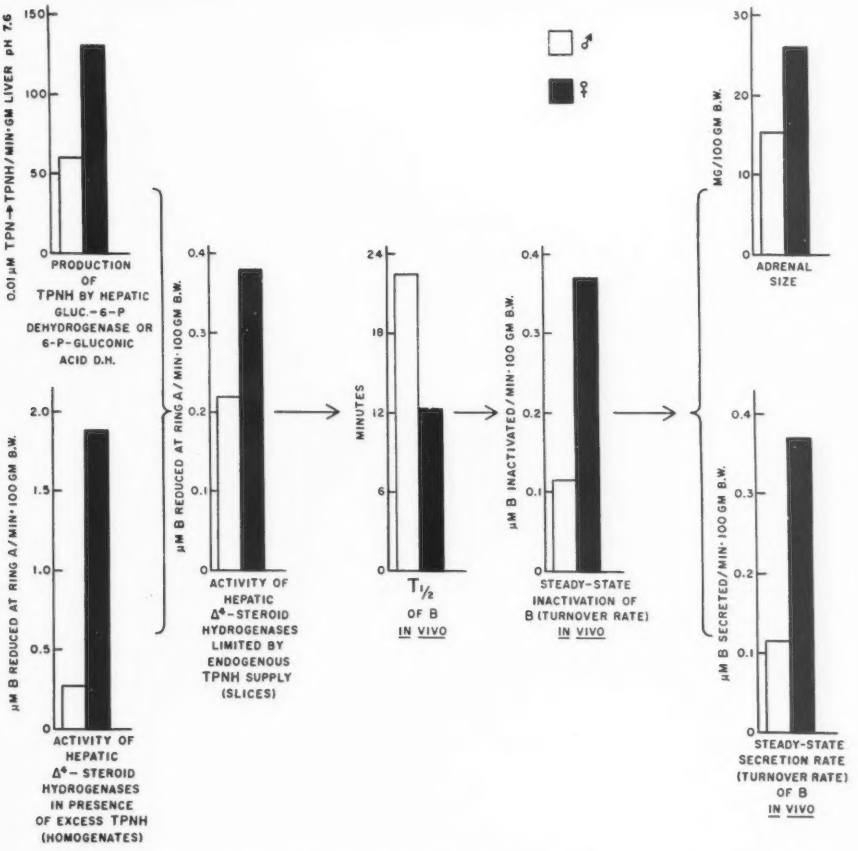
0.01 μM TPN→TPNH/MIN·GM LIVER pH 7.6
PRODUCTION OF TPNH BY HEPATIC GLUC.-6-P DEHYDROGENASE OR 6-P-GLUCONIC ACID D.H.
μM B REDUCED AT RING A/MIN·100 GM B.W.
ACTIVITY OF HEPATIC Δ4-STEROID HYDROGENASES IN PRESENCE OF EXCESS TPNH (HOMOGENATES)
μM B REDUCED AT RING A/MIN·100 GM B.W.
ACTIVITY OF HEPATIC Δ4-STEROID HYDROGENASES LIMITED BY ENDOGENOUS TPNH SUPPLY (SLICES)
MINUTES
T½ OF B IN VIVO
μM B INACTIVATED/MIN·100 GM B.W.
STEADY-STATE INACTIVATION OF B (TURNOVER RATE) IN VIVO
MG/100 GM B.W.
ADRENAL SIZE
μM B SECRETED/MIN·100 GM B.W.
STEADY-STATE SECRETION RATE (TURNOVER RATE) OF B IN VIVO
♂
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FIG. 5. Summary of sex differences in adrenal cortical function in adult rats. The data from the liver assays have been previously reported (1, 21, 23). The arrows indicate our interpretation of the causal relationships among these sex differences. The symbol *B* stands for corticosterone.

tion, which then acts through the negative feedback control of adrenal cortical secretion to cause a greater steady-state secretion rate from the female adrenal cortex. Since this greater secretion rate of the female adrenal presumably requires a greater output of ACTH in the female (or a greater sensitivity of the female adrenal to ACTH) the female adrenal becomes and remains larger than that of the male as soon as the sex difference in hepatic Δ^4-steroid hydrogenases develops (1).

It is not necessary to attribute great importance to sex differences in adrenal cortical physiology, *per se*. However, the sex differences described above have served as a useful means to analyze the interrelations between various components of the adrenal cortical system, without disturbing the

system itself, and they may indicate properties of the system also present in other species, such as man, which do not show prominent sex differences in adrenal cortical function.

Acknowledgments

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LOCAL AND SYSTEMIC EFFECTS OF INTRATESTICULAR TESTOSTERONE AND ESTRADIOL PELLETS IN IMMATURE RATS[1]

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ABSTRACT

Testosterone pellets were implanted into testes of immature intact and of hypophysectomized rats. Ten to twenty days later testes with testosterone pellets were larger than the contralateral testes, confirming earlier reports of a local maintenance effect. When testosterone-treated gonads were compared with initial and terminal controls, it was obvious that testosterone retarded regression of testes in hypophysectomized rats, but also retarded growth of testes in intact rats. Absorption of hormone from 15% testosterone pellets was more rapid during the first 10 days after implantation. Estradiol (5%) pellets had no localized effect when placed in testes of intact rats. In control experiments testosterone, or estradiol pellets were used in the rectus abdominis, or cholesterol pellets in the testis.

EXOGENOUS androgens have been shown to exert ambivalent effects on the testes. Low doses indirectly inhibit spermatogenesis in normal rats by suppression of pituitary gonadotrophins, whereas, large doses maintain spermatogenesis in the hypophysectomized animal, provided the rat is hypophysectomized after 29 days of age (Cutuly, Cutuly, and McCullagh, 1938; Leathem, 1942). High doses of testosterone, which also inhibit the pituitary, result in a level of androgen which maintains testicular weight, the seminiferous tubules, and spermatogenesis (Selye, 1941). In testes containing pellets of testosterone, the high local concentration of androgen maintains spermatogenesis and prevents atrophic changes (seen in the contralateral testes) resulting from subcutaneous administration of low doses of androgen (Ludwig, 1950).

The present experiments were intended to determine the local effect of intratesticular testosterone pellets on the testes of intact and hypophysectomized Sprague-Dawley immature rats. It was also attempted to determine what local effect, if any, estrogen pellets would exert in the testis. Testosterone pellets were implanted intratesticularly in immature rats hypophysectomized at age 25 days to determine to what degree the testis tubules could be maintained or stimulated.

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[2] Recipient of the Borden Award for meritorious research completed during undergraduate medical training.

MATERIALS AND METHODS

All rats were purchased from Sprague-Dawley, Inc. The hypophysectomized rats were Sprague-Dawley rats purchased through Hormone Assay Labs., Inc., Chicago. The hypophysectomized rats were given 2.5 mg. cortisone acetate subcutaneously one day prior to pellet implantations. Otherwise, all animals were maintained on tap water and laboratory chow ad libitum.

Initial control animals were untreated rats, sacrificed at the time pellet implantations were performed in each series. Terminal control animals were untreated rats sacrificed at the termination of each experiment.

Pellets for intratesticular implantation were prepared from the following mixtures: testosterone 15% with cholesterol excipient; estradiol 5% with cholesterol excipient; and cholesterol 100% used as an inert control pellet. The testosterone mixture was prepared by repeatedly mixing and cutting the constituents with a spatula. The estradiol mixture was prepared by dissolving the estradiol in 95% alcohol, mixing with cholesterol, and drying. The pellets were formed by pressing singly in a pellet mould, to a degree of hardness that permitted handling with a thumb forceps. Testosterone pellets weighed 6.0–10.9 mg.; estradiol pellets weighed 5.0–13.4 mg. All pellets were sterilized with dry heat at 100° C for one hour.

A small amount of pre-anesthetic Nembutal was injected intraperitoneally to relax each animal. This procedure reduced the amount of ether required for anesthesia. Post-operative recovery time was 20–30 minutes.

Each pellet was inserted through a small horizontal slit in the tunica albuginea at the mid-portion of the testis. No sutures were required in the tunica albuginea, and no adhesions formed. Testes were palpated daily to judge size, trauma and position. In some animals pellets were placed in the rectus sheath.

All rats were sacrificed by asphyxiation with natural gas, and then weighed. Testes, epididymides, seminal vesicles, ventral prostates and dorsal prostates were dissected, then weighed on a Roller-Smith torsion balance. Testes were fixed in Bouin's solution. Microscopic sections were cut at 5 μ at the level of the pellets, and stained with hematoxylin and eosin.

Weight loss of pellets was not measured in this series of experiments.

OBSERVATIONS

Twelve rats, comprising the first series, were hypophysectomized at 25 days of age. Testosterone pellets were implanted in the right testes of 6 of these rats at 30 days of age (average weight 53 gm.; range 50–56 gm.). All rats were autopsied at 40 days of age (average weight 55–57 gm.).

The right testes with the testosterone pellets averaged 108 mg. in weight, as compared to 57 mg. for the left testes, which were in the same weight range as those of the untreated control animals. Seminal vesicles and ventral prostates of the testosterone treated animals were significantly heavier than those of the controls. This difference can be attributed to a systemic effect of testosterone from the pellet. There was a slight difference in right and left seminal vesicle weights, suggesting a possible selective androgenic action on the side of the pellet.

The testes with testosterone implants contained larger tubules than the contralateral and terminal control testes. The tubules in the latter two groups were approximately equal in size. There were more primary sper-

matocytes in the treated testes than in the contralateral testes or those of the terminal controls. An attempt was made to quantitate this difference by counting the primary spermatocytes per 10 tubular cross-sections, with the following results: testes with pellets, 101; contralateral testes, 26; terminal control testes, 16. The interstitial tissue was atrophic in all testes.

The absence of any intact controls made it impossible to judge whether the testosterone pellet stimulated an increase in testis weight, or whether it retarded the rate of regression resulting from hypophysectomy.

Seven groups constituted the second series. There were 18 intact controls, 18 hypophysectomized, and 6 sham-operated at 25 days of age. At 28 days of age testosterone pellets were inserted into the right testes of 7

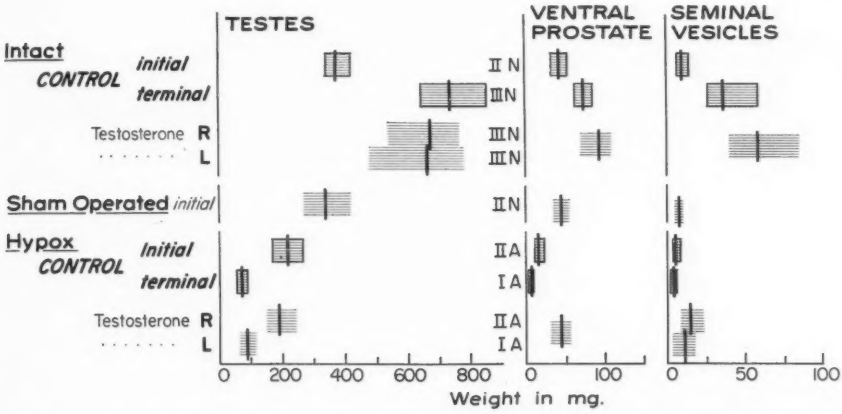

FIG. 1. Organ weights of immature male rats. Some were hypophysectomized at 25 days of age. Testosterone (15%) pellets implanted into right testes at 28 days of age. Autopsies performed at 38 days of age. Histology of the testes is indicated in the column to the right of the testis weights, as follows: I, primary spermatocytes; II, secondary spermatocytes; III, sperm heads; A, atrophic interstitium; N, normal interstitium.

hypophysectomized and 7 intact rats. On this date 5 intact, 6 sham-operated, and 5 hypophysectomized rats were sacrificed as initial controls.

It was observed that by the sixth postoperative day (age 34 days), testes were descended bilaterally in all intact animals, whether treated or controls. Testes were uniformly undescended in hypophysectomized control rats. Of the treated hypophysectomized group, all pellet bearing testes were descended and larger than the untreated testes. Two of the untreated testes were descended, though small; 4 were in the inguinal canal; and one was non-palpable.

Ten days after pellet implantations, the remaining four groups of rats were autopsied. The results of this experiment are summarized in Figure 1. When the five groups of controls were compared, several trends were ap-

parent. The testis weights of the initial intact controls averaged 378 mg. as compared to 739 mg. for the terminal intact controls. The testes of sham-operated controls were somewhat smaller than those of the initial intact controls, while those of the initial hypophysectomized controls had distinctly lost weight. Testosterone pellets seemed to retard the growth of the testes in the intact animals, whereas, they tended to maintain the testes in the hypophysectomized rats. In the intact rats the body weights and testis weights were almost doubled (average body weight gain 63 gm.). The hypophysectomized rats gained an average of 6 gm., but the weights of the untreated testes decreased by more than one-half.

Systemic effects of the testosterone pellets were reflected in the heavier ventral prostates and seminal vesicles. In hypophysectomized rats the right seminal vesicle weights tended to be heavier in the presence of pellets in the right testes.

The controls in this experiment were adequate to show the progressive decline in sexual organ weights after hypophysectomy. Furthermore, the presence of a testosterone pellet in the testis retarded this decline. The effect of the testosterone was localized to the right testes, since the left testes declined as much as those of the hypophysectomized terminal controls.

In the intact rats there was no appreciable effect of testosterone on interstitium or spermatogenesis, since sperm heads were present in terminal controls as well as in treated testes. In hypophysectomized rats the interstitium became atrophic, but testosterone maintained secondary spermatocytes in the tubules. There was no demonstrable localization of effect adjacent to the pellet as claimed by Ludwig and others.

Intact rats were used in the third series, and pellets of testosterone or estradiol were implanted into the testes or into the rectus abdominis muscle as noted in Figure 2. Autopsies were performed at 24 days of age for the initial controls (average weight 61 gm.), and at 36 days of age for the remainder (average weight 117.6 gm.). The testes were growing rapidly during this experimental interval, since the average weight of the initial controls was 218 mg. as compared to 682 mg. for the terminal controls. A testosterone pellet in the rectus muscle prevented any growth of the testes; a pellet in the right testis stimulated or permitted it to increase its weight by 100%; whereas, the left testis was only 50% heavier than the initial control. The presence of an estradiol pellet in the left testis or in the rectus sheath reduced the effect of the testosterone pellet in the right testis.

Ventral prostates were heaviest in rats with testosterone pellets. The seminal vesicle weights correlated closely with the ventral prostate weights as indicators of the systemic effects of testosterone.

Microscopic sections were made for the group with unilateral intratesticular testosterone pellets, and for the terminal control group. Spermiogenesis had progressed to sperm head formation in all sections. Per-

centages of tubules containing sperm heads were determined by counting 2000 tubules in each group: treated testes, 33.1%; contralateral testes, 18.1%; and control testes, 26.0%. Although the pellet bearing testes weighed less on the average than the control group, there was a stimulation of sperm head formation. Conversely, there was inhibition of sperm head formation in the contralateral testes. The interstitial tissue showed no recognizable difference between pellet-bearing and control gonads.

The fourth series consisted of seven groups of intact rats. Testosterone, estradiol, and cholesterol pellets were implanted intratesticularly and/or intramuscularly, as indicated in Fig 3. There were 4 initial controls and 6

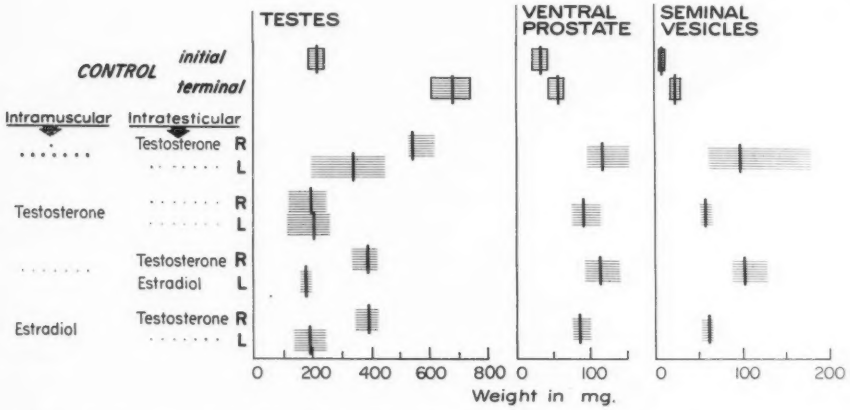

FIG. 2. Organ weights of immature intact male rats. Intratesticular and intramuscular pellets of 15% testosterone and 5% estradiol were implanted at 24 days of age. Autopsies performed at 36 days of age.

terminal controls. Each treated group consisted of 5 rats. Pellet implantations and initial control autopsies were performed at age 40 days (average weight 129.5 gm.; range 112–148 gm.). All remaining rats were autopsied at age 61 days. Weight increments in the treated animals were greatest in the group with intratesticular testosterone plus intratesticular cholesterol pellets (105 to 115 gm.); least in the group with an estradiol pellet in the rectus sheath (59 to 80 gm.). None of the treated animals gained as much weight during the experiment as did the terminal controls (average 128 gm.).

Rats treated with testosterone pellets alone, or in combination with cholesterol pellets, had testis weights which were intermediate between initial and terminal control testis weights. The effect of an intramuscular estradiol pellet on testis weight was not significantly different from that of an unilateral estradiol intratesticular pellet. Each one prevented testis growth. When both estradiol and testosterone were implanted intrates-

ticularly, the estradiol treated testes regressed to almost one-half the initial control weight, and the testosterone bearing testes remained at initial control level.

Ventral prostates in testosterone or testosterone-cholesterol treated rats approached the terminal control level. Ventral prostate weights in all rats which received estradiol approximated those of the initial controls. Seminal vesicle weights were closely correlated with ventral prostate weights, as in the previous series.

Initial control testes contained sperm heads. The only significant effect on spermatogenesis occurred in testes bearing estradiol pellets. These testes

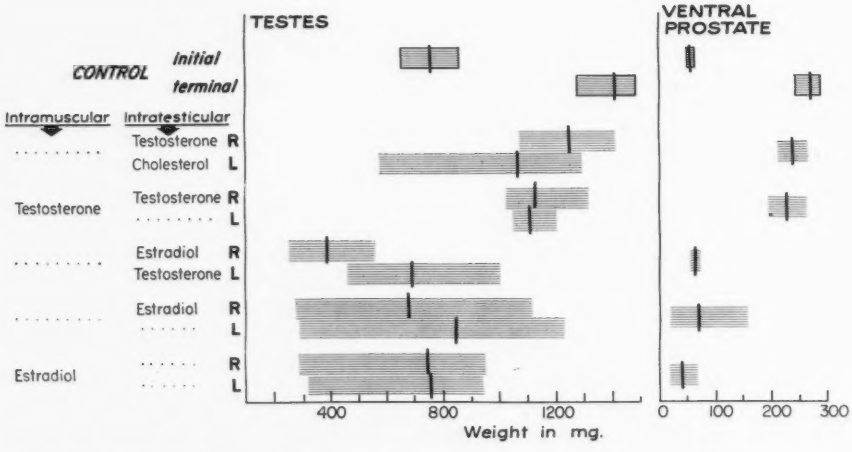

FIG. 3. Organ weights of young mature male rats. Testosterone (15%) and estradiol (5%) pellets implanted at age 40 days. Autopsied at 61 days of age.

contained many degenerating germinal cells, and secondary spermatocytes represented the most advanced stage of maturation.

No local effect could be ascribed to the estradiol pellets. Although each estradiol treated testis was lighter in weight than the opposite untreated or testosterone treated testis, there was no consistent histologic change due to estrogen. The only constant finding was that the number of tubules with degenerating cells was closely correlated with the weight of the testis. The testes with good maintenance of spermatogenesis were invariably heaviest.

DISCUSSION

Evidence for androgenic influence in descent of the testes in hypophysectomized rats was apparent in the following observations: none of the testes of initial and terminal control animals were descended; all testes bearing testosterone pellets descended, even though they did not increase

in size over initial controls; six of the seven contralateral testes were descended or in the inguinal canal, even though their weights regressed to terminal control values.

Burrows (1949) injected intact rats with gonadotrophins, and concluded that size and turgidity of the testes were important factors in favoring descent. Hamilton (1938) found that testosterone induced testicular descent in immature monkeys, and concluded that descent produced by anterior pituitary-like substances was partially due to stimulation of androgen production. Our observations indicate that androgenic effects are more significant than size or turgidity of testes in promoting testicular descent in the rat.

Intratesticular pellets of 15% testosterone stimulated growth of the seminal vesicles and ventral prostates in 10-day experiments, but not in the 21-day experiment (Series IV). Dvoskin (1944) presented evidence for most rapid absorption of testosterone from pellets during the initial 10-day experimental interval, with decreasing rate of absorption thereafter. He observed a greater systemic effect in rats treated for 20 days than in those autopsied 40 days after pellets were implanted. The apparent absence of systemic effect of 15% testosterone 30 days after implantation in Ludwig's experiments (1950) is in accord with Dvoskin's observations of decreasing rate of absorption.

Ludwig observed interstitial atrophy in testes with 100% testosterone implants. However, the interstitium appeared normal in testes treated with 15% testosterone pellets for 30 days. Our series of experiments confirmed the latter observation 10 days after pellet implantations. Scattered interstitial cells appeared atrophic in the presence of 5% estradiol pellets (Series IV), whether the pellets were implanted intratesticularly or intramuscularly. This incipient interstitial atrophy (estrogen induced) was also present in contralateral testes bearing intratesticular testosterone pellets.

Individual testis weight was not correlated with individual body weight within a given series. However, there was a close correlation between tubular size and testis weight, as well as a negative correlation with cellular degeneration. Advanced stages of spermatogenesis were associated with larger tubules, fewer degenerating germinal cells, well maintained interstitium, and heavier testes. Histologic degeneration and weight loss in testes of 28-day-old rats three days after hypophysectomy (Series II) showed the early dependence of immature rat testes on hypophyseal gonadotrophins.

Chedid (1948) implanted testosterone into testes of immature rats. He claimed an increase in testicular weight due to the presence of the testosterone. His experiments did not include initial controls. Our experiments would have the same apparent results if only the terminal controls were considered. This type of experiment requires two sets of controls. His

claims for precociously induced spermatogenesis also would require the additional set of controls.

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EFFECTS OF OVARIECTOMY ON THE FOLLICLE-STIMULATING AND INTERSTITIAL-CELL-STIMULATING CONTENT OF THE ANTERIOR PITUITARY OF THE RAT[1]

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ABSTRACT

Anterior pituitaries from normal rats and ovariectomized rats of various postoperative ages have been assayed in immature hypophysectomized female rats for follicle-stimulating (FSH), interstitial-cell-stimulating (ICSH) and growth-stimulating (GH) activity. Histological criteria have been used to determine FSH and ICSH activities, and width of the tibial epiphyseal cartilage plate used as an index of GH activity.

The pituitary FSH content increased markedly (3- to 4-fold) within 7 days following ovariectomy and continued to increase progressively when tested at 2 weeks, one month and 2 months postoperative. No further increase was found after a 4 or 9 month period. The pituitary ICSH content likewise showed a marked increase (approximately 4 times the normal level) at 7 days postoperative, and continued to increase progressively up to 9 months, the longest period tested. GH content remained the same in pituitaries of both normal rats and ovariectomized rats for 2 months after the operation, but a slight decrease in GH content was found with a 4 and 9 month postoperative period.

THE marked increase in gonadotrophic content of the anterior pituitary of the rat following castration has long been established, e.g., Evans and Simpson (1) Engle (2). Most of the early reports were concerned with follicle-stimulating activity alone or with the gonadotrophic complex as a whole, so there has been considerable disagreement as to whether the increased content was due primarily to follicle-stimulating or luteinizing activity. More recently, attempts have been made to differentiate between follicle-stimulating (FSH) activity and interstitial-cell-stimulating (ICSH) or luteinizing (LH) activity, but the results and interpretations have varied with the bioassay methods used, the length of the postoperative period, and the sex of the donor rats.

The present study was undertaken to investigate the FSH and ICSH content of the anterior pituitary of ovariectomized rats and to determine what changes in content take place with a progressive increase in the postoperative period. In addition, growth-stimulating (GH) activity in the anterior pituitary following gonadectomy has been investigated.

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MATERIALS AND METHODS

Virgin female rats of the Long-Evans strain averaging 100 days of age and 220 grams in body weight were bilaterally ovariectomized and maintained on a stock diet of natural foodstuffs[2] for postoperative periods of one week, 2 weeks, and one, 2, 4, and 9 months. Normal female rats from 80 to 160 days of age at time of autopsy were used as controls.

The methods used for bioassay of the pituitaries were those of Wooten *et al.* (3). Anterior pituitaries of donor animals were dissected, weighed, ground with mortar and pestle, and suspended in isotonic saline containing 2% butanol. Pituitaries from 25 to 40 or more rats from each postoperative period were pooled in groups of 6 to 12 glands and the suspensions stored at 4° C prior to assay.[3] The completeness of ovariectomy was verified by direct inspection of the operation site at the time of sacrifice. Glands from over 500 normal female rats were pooled in groups of 25 to 60 for the control bioassays. Immature hypophysectomized female rats 26 to 29 days of age at operation and 11 to 21 days postoperative were used as test animals. Pituitary suspensions were diluted with isotonic saline and injected as fractions or multiples of glands at dosage levels varying from $\frac{1}{3\frac{1}{2}}$ to 4 glands (total dose). Injections were given subcutaneously once daily for 4 days. Three to 5 test animals were used for each dosage level and all levels were repeated one or more times. Autopsy was performed 24 hours after the last injection. The ovaries were dissected free of the oviducts, weighed and fixed in Bouin's fluid for histological study, the uteri (minus the oviducts) drained of fluid and weighed, and the right tibiae split midsagitally, fixed in formol and stained with $AgNO_3$. Completeness of hypophysectomy was verified at autopsy by examination of the pituitary site under the binocular microscope.

The criteria described by Evans *et al.* (4) were used to detect the histological responses for FSH and ICSH in the ovaries of the test animals. The minimal effective dose (MED) for FSH was that fraction of a gland or number of glands necessary to cause beginning antrum formation and to increase the size of follicles in $\frac{2}{3}$ of the ovaries of the test animals. Follicles were measured with a calibrated eyepiece micrometer (see footnote 3, Table 1). The MED for ICSH was that fraction of a gland or number of glands necessary to cause resumption of the normal nuclear pattern in "deficient" interstitial tissue with some increase in cytoplasm in $\frac{2}{3}$ of the ovaries of the test animals. The standard tibia test (5) was used to detect the presence of GH activity. The uncalcified portion of the proximal epiphyseal cartilage was measured with a micrometer eyepiece and an average width of 200 micra or more (an average increase of 35% over the uninjected control values) was considered evidence for GH activity.

RESULTS

Table 1 summarizes the results of the bioassays for FSH and ICSH activity in the anterior pituitaries of normal and gonadectomized female rats. FSH activity was first detected in the glands of normal female rats at the 3 pituitary dosage level. Since some ovaries contained medium and large follicles this was considered a response greater than the MED. However, at the 2 pituitary dosage level only small follicles were found so the MED for FSH activity was considered to fall between 2 and 3 glands for the normal animals.

[2] Stock diet XIV is composed of 68.5% ground whole wheat, 10% fish meal, 10% alfalfa leaf meal, 5% casein, 5% fish oil (Sardilene) and 1.5% iodized NaCl. Lettuce given ad libitum twice weekly.

[3] According to Wooten *et al.* (3), pituitary suspensions with butanol added as a preservative, can be stored for 60 days without detectable loss in gonadotropic activity as judged by bioassay.

TABLE 1. FOLLICLE-STIMULATING AND INTERSTITIAL-CELL-STIMULATING ACTIVITY IN THE ANTERIOR PITUITARIES OF NORMAL AND OVARIECTOMIZED RATS

Total pituitary dosage		Test animals[1]	Uterus wt.[2]	Ovaries			Activity[4]	
	Wt			Wt	Follicles[3]	Interstitial tissue	FSH	ICSH
no.	mg.	no.	mg.	mg.				
					Normal			
0	0	37	15	8	s	deficient	Neg.	Neg.
½	5.0	28	18	8	s	deficient	Neg.	Neg.
1	10.1	33	18	10	s	partial repair	Neg.	MED
2	20.0	33	21	12	s	repair	Neg.	>MED
3	29.9	20	30	16	sm–m	repair	>MED	>MED
4	40.5	19	52	32	m–l	repair	>MED	>MED
					Castrate—One week post-operative			
0	0	13	16	8	s	deficient	Neg.	Neg.
¼	1.1	15	17	9	s	deficient—partial repair	Neg.	<MED
½	2.1	15	19	10	s	partial repair	Neg.	MED
¾	4.2	12	20	11	s	partial repair—repair	Neg.	>MED
1	8.6	14	93	35	m–ml	repair	>MED	>MED
					Castrate—Two weeks post-operative			
0	0	12	15	7	s	deficient	Neg.	Neg.
¼	1.2	11	15	8	s	deficient	Neg.	Neg.
½	2.3	11	18	11	s	partial repair (all test rats)	Neg.	>MED
¾	4.7	11	29	17	sm	repair	MED	>MED
1	8.6	7	68	47	m–l, CL	repair	>MED	>MED
					Castrate—One month post-operative			
0	0	15	16	7	s	deficient	Neg.	Neg.
1/32	0.3	8	16	9	s	deficient	Neg.	Neg.
1/16	0.7	12	15	9	s	deficient—partial repair	Neg.	<MED
¼	1.3	13	17	10	s	partial repair	Neg.	MED
½	2.5	10	17	10	s	repair	Neg.	>MED
¾	4.8	9	37	20	sm, few m	repair	>MED	>MED
1	10.1	7	60	49	m–l, CL	repair	>MED	>MED
					Castrate—Two months post-operative			
0	0	17	16	8	s	deficient	Neg.	Neg.
1/32	0.3	11	17	8	s	deficient	Neg.	Neg.
1/16	0.6	12	17	9	s	deficient—partial repair	Neg.	<MED
¼	1.2	10	16	11	s	partial repair+repair	Neg.	>MED
½	2.4	11	38	18	sm, few m–l	repair	>MED	>MED
¾	4.8	9	74	72	m–l , CL	repair	>MED	>MED
1	9.5	10	68	68	m–l, CL	repair	>MED	>MED
					Castrate—Four months post-operative			
0	0	6	15	7	s	deficient	Neg.	Neg.
1/32	0.4	7	16	9	s	deficient	Neg.	Neg.
1/16	0.7	13	16	10	s	partial repair	Neg.	MED
¼	1.4	12	18	12	s–sm	repair	<MED	>MED
½	2.8	13	41	19	sm–m	repair	>MED	>MED
¾	5.6	13	66	50	m–l, CL	repair	>MED	>MED
1	11.3	7	64	51	m–l, CL	repair	>MED	>MED
					Castrate—Nine months post-operative			
0	0	10	14	8	s	deficient	Neg.	Neg.
1/32	0.4	10	14	9	s	deficient—partial repair	Neg.	<MED
1/16	0.7	10	15	12	s	partial repair (all test rats)	Neg.	>MED
¼	1.4	10	33	16	s–sm	repair	<MED	>MED
½	2.8	9	55	34	m–ml, few l, CL	repair	>MED	>MED
¾	5.6	4	74	65	m–l, CL	repair	>MED	>MED
1	11.3	3	68	74	m–l, CL	repair	>MED	>MED

[1] Female rats hypophysectomized 26–29 days of age, 11–19 days post-operative.
[2] Oviduct removed and uterus drained of fluid.
[3] Abbreviations: CL = corpora lutea; s = small, m = medium, l = large—refer to size of follicles measured with calibrated eyepiece micrometer. Standards used by Wooten *et al.* (3). s = up to 375 μg, sm = 450–500 μg, m = 550–650 μg ml = 700–750 μg, l = 800–1000 μg.
[4] See text for explanation of MED (minimal effective dose).

Seven days after gonadectomy FSH activity was observed at a lower dosage level (one pituitary) but here again the response was in excess of the MED since medium and medium-large follicles were present in the ovaries. Decreasing the dosage level to ½ pituitary resulted in small follicles only, so the MED for FSH in rats one week postoperative was considered to be between ½ and one pituitary. With a postoperative period of 2 weeks a further increase in pituitary FSH content was indicated by an MED of ½ gland. The FSH content continued to increase as is shown by an MED of ¼ to ½ gland for a one month postoperative period and an MED

between $\frac{1}{8}$ and $\frac{1}{4}$ gland for 2 months postoperative period. No further increase in FSH activity occurred with the longer postoperative intervals of 4 and 9 months.

It should be noted that increases in uterine and ovarian weights accompanied the increased follicular development observed histologically and corpus luteum formation occurred with the higher pituitary dosage levels (e.g., one gland) of all postoperative intervals except that of one week. Additional tests (not included in Table 1) have shown that with the short postoperative period of one week a total dosage of 3 to 4 glands was usually required for formation of corpora lutea.

TABLE 2. GONADOTROPHIC ACTIVITY IN THE ANTERIOR PITUITARIES OF NORMAL AND OVARIECTOMIZED RATS

Donors, length of post-oper. period	FSH activity MED,[1] No. pits	ICSH activity MED,[1] No. pits
0	2–3	1
One week	$\frac{1}{2}$–1	$\frac{1}{4}$
Two weeks	$\frac{1}{2}$	$\frac{1}{8}$ – $\frac{1}{4}$
One month	$\frac{1}{4}$–$\frac{1}{2}$	$\frac{1}{8}$
Two months	$\frac{1}{8}$–$\frac{1}{4}$	$\frac{1}{16}$–$\frac{1}{8}$
Four months	$\frac{1}{8}$–$\frac{1}{4}$	$\frac{1}{16}$
Nine months	$\frac{1}{8}$–$\frac{1}{4}$	$\frac{1}{32}$–$\frac{1}{16}$

[1] See text for explanation of MED (minimal effective dose).

ICSH activity was first detected in normal glands at the one pituitary dosage level and this was considered the MED since in the majority of test animals the ovaries showed partial repair of the "deficient" interstitial tissue. One week following ovariectomy, a 4-fold increase in ICSH content was noted as injection of $\frac{1}{4}$ gland resulted in the same histological response. The ICSH content of the pituitary increased progressively with each postoperative period up to 9 months, the longest period tested. Table 2 summarizes the pituitary dosage levels required for the MED in each postoperative period and shows the increase in both FSH and ICSH content as the postoperative interval increases.

GH activity was present in equivalent amounts in the glands of normal donors and in those from donors gonadectomized for one week, 2 weeks, one month and 2 months (Table 3). In these groups $\frac{1}{16}$ of a gland (total dose) produced a significant increase in the epiphyseal cartilage widths of the test animals. With the longer postoperative periods of 4 and 9 months, there appeared to be a slight decrease in GH content.

DISCUSSION

The data presented show a simultaneous increase in the FSH and ICSH content of the anterior pituitary as early as 7 days after ovariectomy and a progressive increase in activity when the postoperative period was extended to two months. After this period, the FSH content remained un-

changed whereas the ICSH content continued to increase up to 9 months, the longest postoperative period tested.

The marked increase in ICSH content of the pituitary during the first two weeks following ovariectomy is similar to that reported recently by Parlow,[4] who used depletion of ovarian ascorbic acid to determine ICSH or LH activity. Other investigators (6, 7), who used corpus luteum formation as the criterion for LH, have reported that the pituitary during the first fifteen days following gonadectomy of either male or female rats was predominantly follicle-stimulating, followed by a period lasting up to 9 months in which luteinizing activity was predominant.

TABLE 3. PROXIMAL EPIPHYSEAL CARTILAGE WIDTHS[1] FROM TIBIAE OF IMMATURE HYPOPHYSECTOMIZED RATS INJECTED WITH ANTERIOR PITUITARY SUSPENSIONS

Donors, post-operative period	Recipients[2]						
	Fraction of anterior pituitary injected						
	0	$\frac{1}{32}$	$\frac{1}{16}$	$\frac{1}{8}$	$\frac{1}{4}$	$\frac{1}{2}$	1
0	145 ± 5[3]	181 ± 8	221 ± 11	238 ± 10	264 ± 11	281 ± 9	306 ± 12
One week	152 ± 5	187 ± 12	211 ± 9	235 ± 9	259 ± 9	279 ± 11	297 ± 13
Two weeks	156 ± 7	201 ± 12	201 ± 10	241 ± 13	273 ± 10	293 ± 9	280 ± 15
One month	154 ± 6	191 ± 9	228 ± 7	251 ± 10	271 ± 11	284 ± 14	332 ± 17
Two months	154 ± 6	190 ± 12	201 ± 12	209 ± 19	250 ± 15	269 ± 13	32 ± 25
Four months	163 ± 9	185 ± 8	189 ± 15	243 ± 11	252 ± 12	286 ± 10	271 ± 15
Nine months	130 ± 8	162 ± 9	177 ± 4	172 ± 9	232 ± 11	210 ± 26	222 ± 7

[1] Uncalcified portion of cartilage was measured and average widths of 200 micra or greater were considered evidence for growth-promoting activity (GH).

[2] Female rats hypophysectomized 26–29 days of age, 11–21 days post-operative. The maximum number of test animals per group is given in Table 1.

[3] S.E. $= \sqrt{\frac{d^2}{n(n-1)}}$

The ICSH content of the pituitaries of normal adult female rats reported by Wooten *et al.* (3) is the same as that observed in the present study but the FSH content was significantly less than reported here. In view of the fact that identical procedures were used in both investigations, it was thought that this difference might be due to variation in FSH content with stages of the estrous cycle. This possibility was tested by pooling glands from rats at 4 stages of the cycle as judged by vaginal smear, i.e., proestrus, estrus, post-estrus and diestrus. Several bioassays at each stage of the cycle were carried out, but no consistent differences in FSH potency dependent on the cycle were found.

The GH content of the anterior pituitary was investigated because of the conflicting reports on the effects of castration on the acidophils (see review by Severinghaus (8)). In agreement with other investigators (9, 10) who assayed pituitaries of male rats one to 2 months postoperative, we

[4] Presented at the 41st meeting of the Endocrine Society, Atlantic City, New Jersey, 1959.

found no significant change in pituitary GH content in female rats for the first 2 months following ovariectomy. The slight decrease in GH content with longer postoperative periods (4 and 9 months) observed in this study will need confirmation. No significant change in growth-promoting activity of the plasma has been reported following gonadectomy of either male (10) or female (11) rats.

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INHIBITION OF ADRENAL CORTICOSTEROID RELEASE BY BRAIN STEM STIMULATION IN CATS[1]

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ABSTRACT

Facilitatory and inhibitory components of the nervous system have been postulated for the regulation of ACTH. Some of these have been activated in the present study by electrical stimulation in two brain stem areas in cats prepared as encéphale isolés. 30 second stimulation in the ventral midbrain tegmentum evoked a specific and rapid decrease in adrenal effluent corticosteroids of 7 cats; stimulation in the posterior diencephalon in 17 cats was followed by an increase, a decrease or no significant change in corticosteroid levels. An interrelationship between midbrain and diencephalic areas concerned with the regulation of ACTH release appears evident on the basis of reversals in corticosteroid levels in response to combined stimulation. That the inhibitory and facilitatory effects on corticosteroid release are neurological in origin rather than due to alteration of peripheral mechanisms seems evident on the basis of the rapidity of response and the lack of correlation with blood pressure changes.

A NUMBER of areas in the central nervous system have been demonstrated or hypothesized to play a role in the regulation of ACTH release by the adenohypophysis. Substantial evidence in recent years, based on experiments in which the hypothalamus had been stimulated or lesioned, revealed a facilitatory role of this area. Stimulatory factors have also been demonstrated in the amygdala (1, 2) and postulated for the hippocampal cortex (3). Evidence is scarce for specifically localized areas in the central nervous system involved in inhibition of the pituitary-adrenal axis. Stimulation of the uncus (3) as well as hippocampal structures (4) have indicated inhibitory phenomena associated with corticotropin release. Neuronal inhibition of ACTH from the midbrain is thus far based on evidence from the effects of lesions in this area (5, 6).

The present experiments were designed to determine whether ACTH release, as measured by adrenal effluent corticosteroids, could be inhibited by electrical stimulation of the midbrain in the unanesthetized cat. In addition an attempt has been made to demonstrate on a neuroendocrine basis

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an interaction between the midbrain and diencephalon in the regulation of ACTH release.

MATERIALS AND METHODS

30 adult male cats, 2–4 kg., were prepared as encéphale isolés under ether by the method of Bremer (7). Following craniotomy for electrode placement, and spinal cord section between C-1 and C-2, ether administration was discontinued and artificial respiration instituted. A rectal thermometer was inserted and heat lamps adjusted for maintenance of body temperature.

Twenty to thirty minutes after cord section, laparotomy was performed by making a transverse incision through the abdominal wall just below the rib cage. Retractors were placed permanently over the viscera to expose the left adrenal gland and surrounding vessels. A ligature lift was placed under the adrenal vein in such a manner as to permit continuous blood flow through the gland except during sample collection. A #160 polyethylene tube was inserted and tied in the distal portion of the adrenal vein. Accessory vessels into the adrenal gland were tied off. Immediately post-cannulation an adrenal venous effluent sample was collected into a tube with 0.05 ml. heparin. Blood remaining in the cannula at termination of each collection period was returned to the animal with a minimal amount of heparin (0.2–0.3 ml.). The exposed area was covered with warm salinized gauze and a square of plastic. Two to three additional samples were collected during the first 4 hours after cord section. All adrenal effluent collection periods averaged 5 minutes (range 3–10 minutes).

Following collection of the first effluent sample, silver ball electrodes were placed on the dura over the right suprasylvian gyrus for electrocorticographic (ECG) recording (Offner type D EEG). Bipolar concentric (#26 gauge) electrodes were inserted stereotaxically into the left posterior diencephalon and/or the left ventral midbrain tegmentum (Horsley-Clarke coordinates for diencephalon were A8.5, L1.5 and H-3 mm.; for midbrain, either A3, L3, H-0.5 or A2, L2 and H-2). The two areas stimulated were selected (a) on the basis of previous experiments in rats where lesions in these areas appeared involved in ACTH regulatory mechanisms (5, 6), and (b) on the basis of established oculomotor responses to stimulation (8) to aid in monitoring electrode placement and threshold.

Following electrode placement the right femoral artery and vein were cannulated for manometric recording of arterial blood pressure and for intravenous drip of whole cat blood. Heart rate was recorded as an EKG (Offner EEG) between a leg clip and one cortical lead.

All collections of adrenal venous effluents were protected from light and kept cold. Samples were immediately centrifuged, the plasma withdrawn and frozen. All individual plasma corticoids were measured by chromatographic and colorimetric methods (9) with an additional step to provide more quantitative analysis for low recovery levels: a known concentration of 17-OH corticosterone or corticosterone was added to the appropriate sample eluted from the chromatographic strip. The final colorimetric reading recorded was based on the difference between the known concentration of the standard and the unknown sample. Corticosteroid data are expressed as total μg of steroids recovered from each sample as calculated on a 5 minute collection basis. No attempt has been made in the present study to differentiate between the various adrenal steroids released and a specific neurogenically evoked pituitary-adrenal response.

All stimulations of single brainstem sites were 30 second trains of square waves (Grass S 4 B stimulators) at 25 per second, pulse duration 15 msec., and 3 volts (derived from parameters found effective in eliciting autonomic and eosinopenic responses by Hess (10) and Porter (3)). These parameters have previously been demonstrated to activate neu-

rons within an area not exceeding 1 mm. in diameter (8). Various experimental protocols were utilized (cf. *Results and Discussion*), with at least 3 effluent collections made at intervals for one to two hours after each stimulation. Although ether could be presumed to be dissipated 1 to 2 hours after cord section, no stimulation was applied until 4 hours after section.

The condition of the cat was constantly monitored for blood pressure, heart rate, body temperature, pupillary size and light reflexes, spontaneous and alerting movements of eyes, and electrocorticograph. The experiment was terminated for any cat whose blood pressure dropped below 60 mm. Hg, whose pupillary light reflex was sluggish, who exhibited anisocoria, or whose ECG was abnormal.

Prior to termination of the experiment, 13 cats received 40 U.S.P. units of ACTH i.v. over a 60 second period, and 3 separate samples were collected during the next 20–30 minutes.

Just before the animal was sacrificed with an overdose of barbiturate, each brainstem site was stimulated at 250 per sec., 3 msec., and 1–6 volts to evoke conjugate eye movements (8), as a final check on the animal's condition and to provide an index as to reliability of electrode placement prior to chromatographic analysis of corticosteroids.

Stimulated sites were lesioned (3 m.amps. D.C., 10 sec.) for subsequent histological identification and reconstruction of areas stimulated. Brains were fixed in 10% formalin, sectioned at 80 μ, and stained with thionin.

RESULTS AND DISCUSSION

The encéphale isolé has been utilized in the present study in preference to other acute preparations for the following reasons: (a) Anesthetics are known to depress central neuronal areas involved in the present studies (11, 12); the encéphale isolé requires no further anesthesia than the ether used for preliminary surgery and cord section. (b) The central nervous system is intact above the spinal cord (13); any pressure damage can be detected on the basis of alterations in eye movements (12), pupil size, and cranial reflexes even before deterioration is evident in ECG or blood pressure. (c) Cranial mechanisms permit monitoring of the animal's "central" condition; thus spontaneous eye movements, as well as ECG, reveal alternation of alert and "drowsy" states (7, 14).

A. *Corticosteroid changes in the non-stimulated encéphale isolé cat* (Table 1). In 6 control non-stimulated cats, the maximal percent changes noted in 5 minute adrenal effluent total corticosteroid levels over any two hour period, from 3 to 10 hours after cord section, were +25 and −25%. On this basis only the following results of stimulation have been considered significant: *increased* response if corticosteroid levels were elevated more than 25% during one to two hours post-stimulation; *decreased* response if these values fell below −25%. All percent changes in the experimental series are expressed relative to the last immediate pre-stimulation value, such value equivalent to 100%. Inasmuch as "initial" levels (pre-stimulation) of corticosteroids were variable from cat to cat, each animal served as his own control for relative percent changes.

That levels of corticosteroids recovered did not decrease appreciably during 9 or more hours is in contrast to the observations of Brodish and

TABLE 1. ADRENAL EFFLUENT CORTICOSTEROID LEVELS IN NON-STIMULATED ENCÉPHALE ISOLÉ CATS

Cat No.	Corticosteroids: total μg/5 minutes*										
	Hours post-cord section										
	1–2	2–3	3–4	4–5	5–6	6–7	7–8	8–9	9–10	10–11	11–13
2	29.5	25.0	—†	25.8	19.7	21.3	17.5	21.9	—	—	—
3	20.7	17.2	12.2	14.2	15.8	16.1	—	—	—	—	—
9	9.2	10.1	12.7	11.3	—	—	—	—	—	—	—
12	14.0	15.6	18.0	—	15.8	—	13.2	—	—	16.3	12.2
16	12.6	12.2	11.8	—	9.5	—	8.2	—	10.0	—	—
50	5.3	6.1	7.5	6.5	7.0	5.5	6.4	5.4	—‡		

* Average of 1–3 samples taken during each hourly period noted.
† In this and following tables periods without values represent either "lost" samples or periods omitted in sampling.
‡ Response to ACTH 9 hours after cord section: 9.7 μg/5 min. in cat 50.

Long (15) indicating decrease in ACTH output 6 hours after removal of a stress in rats. The difference may represent a species variation, or it is also possible that the encéphale isolé cats in the present experiments are maintained in a relatively constant stressed state. That the stress state is not maximal in all cats is indicated by those stimulations or injections of ACTH which resulted in corticosteroid output significantly elevated above the initial levels (cf. Table 1, cat 50).

B. *Effect of single midbrain stimulation (30 sec.) on adrenal corticosteroid release* (Table 2). Following stimulation of a site in the ventral midbrain tegmentum 4 hours after cord section, a significant decrease in corticosteroid release was noted in 7 cats. In each animal the first sample collected after stimulation revealed a significant decrease as compared with the pre-stimulation level; corticosteroid levels remained low for at least one hour after stimulation.

The stimulated area is one which had previously been implicated in inhibition of ACTH release in rats: lesions in the ventral midbrain tegmentum at the level of the rostral pons resulted in elevated corticosteroid

TABLE 2. EFFECT OF SINGLE MIDBRAIN STIMULATION (30 SEC.) ON ADRENAL EFFLUENT CORTICOSTEROID LEVELS

Cat No.	Corticosteroids: total μg/5 minutes (single samples)								
	Hours post-section			Minutes post-stimulation				% change	Response to ACTH 40 I.U.†
	1–2	2–3	4‡	15	30	60	120		
14	4.5	3.6	4.2	—	—	*1.8*	2.3	*−54*	—
17	22.2	22.7	24.8	—	*12.8*	—	9.8	*−48*	—
19	—	18.3	16.5	—	*11.8*	8.1	6.2	*−32*	—
37	2.5	2.6	2.9	*0.6*	0.5	—	—	*−71*	—
39	3.5	3.9	4.6	*2.3*	1.6	2.2	2.1	*−50*	3.0
48	3.3	1.4	1.5	*1.0*	0.0	0.1	1.9	*−33*	3.0
52	16.3	9.3	11.8	*5.0*	—	7.9	8.4	*−58*	10.1

Italicized data represent first significant changes from pre-stimulation levels.
‡ Immediate pre-stimulation value.
† U.S.P. Reference Std. ACTH (i.v.).

release levels which were further increased in response to acute stress (5). The midbrain tegmentum is an area through which a variety of fibers pass (16, 17). The specific inhibition of ACTH release observed following a single stimulation in this area in the present experiments may indicate a preponderance of inhibitory fibers (5, 6); it may also be related to the stimulus parameters used (18). Only a random sampling of sites scattered throughout an area occupying approximately 45 cubic mm. was made; there was no attempt to survey the ventral midbrain tegmentum completely.

The significant decrease in corticosteroid levels noted 15 minutes after a

TABLE 3. EFFECT OF SINGLE DIENCEPHALIC STIMULATION (30 SEC.) ON ADRENAL EFFLUENT CORTICOSTEROID LEVELS

Group	Cat No.	Corticosteroids: total μg/5 minutes (single samples)								
		Hours post-section			Minutes post-stimulation				% change	Response to ACTH 40 I.U.†
		1–2	2–3	4‡	15	30	60	120		
A	15	10.8	8.4	9.8	—	12.2	—	*12.8*	*+31*	—
	35	13.2	18.5	18.5	*25.0*	26.4	23.2	35.5	*+35*	12.0
	44	17.9	17.0	17.5	*27.1*	24.1	25.1	—	*+55*	16.5
	36	—	4.1	3.1	3.4	—	*6.5*	2.2	*+110*	4.8
B	43	15.8	16.2	17.2	*10.8*	11.0	9.3	11.6	*−37*	—
	22	—	16.6	16.5	—	*8.2*	8.8	6.7	*−50*	—
	5	14.3	13.2	10.2	—	10.6	—	*7.0*	*−30*	—
C	7	7.8	9.2	10.7	—	10.0	8.4	9.0	(− 7, −22)	—
	46	—	8.8	7.7	7.2	6.3	5.9	6.3	(− 6, −23)	13.5
	6	—	4.4	3.7	—	4.4	—	3.2	(+19, −14)	—

Italicized data represent first significant changes from pre-stimulation levels.
‡ Immediate pre-stimulation value.
( ) Range noted for non-significant changes.
† U.S.P. Reference Std. ACTH (i.v.).

single 30 sec. unilateral stimulation of the midbrain tegmentum would appear to indicate a neural basis for the inhibition of ACTH release. The prolonged depression of corticosteroid levels is not unexpected in view of the multi-neuronal reverberating circuits of the reticular formation, with their long after-discharge (19).

C. *Effect of single diencephalic stimulation (30 sec.) on adrenal corticosteroid release* (Tables 3 and 4). In 17 cats single stimulation of a site in the posterior diencephalon 4 hours after cord section resulted in either increase, decrease or no change in effluent corticosteroid levels when compared with pre-stimulation values. In 6 cats (Tables 3 and 4, Group A), corticosteroid levels were significantly increased; in 3 cats these increases were noted within the first 30 minutes post-stimulation. In 6 cats (Tables 3 and 4, Group B) a decrease was noted; in 4 of these corticosteroid levels had decreased significantly within 30 minutes post-stimulation. In 5 cats (Tables 3 and 4, Group C) corticosteroid levels were not significantly altered during the two hour period after diencephalic stimulation.

The posterior diencephalon is a region of mixed fibers of passage (17, 20);

results of stimulation in this area have been shown to be diverse with respect to autonomic and somatic responses (21, 10). Whether stimulation in this area results in inhibition or in facilitation of ACTH release may depend on the relative distribution of inhibitory and excitatory neurons at the electrode tip, or may depend on the relative threshold of inhibitory and excitatory neurons at the moment of stimulation. The possibility that the relative threshold of different neurons may be influenced by afferents from other parts of the nervous system (4) cannot be excluded in view of the rich diversity of neuronal input into this area.

There are several possible explanations as to why diencephalic stimulation failed to result in a significant alteration of adrenal corticosteroid release in some cats. (a) If the adrenal or the adenohypophysis were already secreting maximally, an excitatory stimulus could produce no further increase. (b) Although histological study of stimulated diencephalic sites revealed random placement of sites whose stimulation resulted in varied responses in corticosteroid release, it is possible that in those cats which showed no alteration from pre-stimulation levels the majority of neurons in the vicinity of the electrode tip were either unrelated to ACTH regulation or were in an area where inhibitory and facilitatory fibers were equally balanced.

D. *Effect of single diencephalic and of combined midbrain-diencephalic stimulation on adrenal corticosteroid release.* In 7 cats, two hours after single 30 sec. diencephalic stimulation, sites in the midbrain and diencephalon were stimulated simultaneously for 30 sec. Data for these cats are presented in Table 4.

In 2 cats (group A) single diencephalic stimulation elicited an increase in adrenal corticosteroid release; combined stimulation of the midbrain and diencephalon reversed the response, depressing levels of steroids.

In 3 cats (group B) single diencephalic stimulation depressed corticosteroid release, while combined stimulation reversed this effect, eliciting a significantly increased release.

In 2 cats (group C) single diencephalic stimulation did not significantly alter corticosteroid levels from those noted during the initial (pre-stimulation) period. After combined stimulation, however, corticosteroid levels were depressed.

There are several possible hypotheses to explain the reversals observed with sequential stimulation. (a) Possibly impulses from the posterior diencephalon could alter the set or threshold of either facilitatory or inhibitory neurons in the midbrain concerned with regulation of ACTH release; it is known that impulses from the posterior diencephalon can exert a functional influence on the midbrain tegmentum (22). (b) Inhibitory or facilitatory influences from other neuronal areas which can influence regulation of ACTH release could feed back into either the midbrain or the diencephalon to influence subsequent stimulation (4). However, the fact

TABLE 4. EFFECT OF SINGLE DIENCEPHALIC, FOLLOWED BY COMBINED MIDBRAIN-DIENCEPHALIC, STIMULATION ON ADRENAL EFFLUENT CORTICOSTEROID LEVELS

Group	Cat No.	Corticosteroids: total μg/5 minutes (single samples)													
		Hours post-section			Minutes post-stimulation Diencephalon (30 sec.)				% change	Minutes post-stimulation Midbrain + Diencephalon				% change	Response to ACTH 40 I.U.†
		1–2	2–3	4‡	15	30	60	120‡		15	30	60	120		
A	24	—	9.2	8.3	—	*13.5*	15.0	15.5	*+63*	—	*4.3*	10.8	12.7	*− 72*	—
	32	17.6	15.0	16.8	18.8	16.7	20.3	*21.3*	*+27*	*4.4*	8.8	19.0	19.5	*− 79*	21.5
B	29	12.4	—	15.1	—	*8.5*	8.7	2.9	*−44*	3.5	*10.3*	14.5	16.2	*+270*	22.1
	30	—	10.2	11.1	10.3	*7.6*	—	7.9	*−32*	*12.7*	11.7	9.3	6.1	*+ 61*	—
	40	4.7	—	5.0	3.8	4.0	4.7	*3.6*	*−28*	4.0	*4.9*	5.2	4.6	*+ 36*	4.9
C	34	9.4	10.6	12.4	10.0	10.9	14.0	10.8	(−19, +11)	*7.9*	8.5	9.4	9.9	*− 27*	7.8
	41	—	6.6	7.5	7.6	7.2	6.9	5.6	(−25, + 1)	*4.0*	5.1	4.1	3.9	*− 29*	4.8

Italicized data represent first significant changes from each pre-stimulation level.
‡ Immediate pre-stimulation value.
( ) Range noted for non-significant changes.
† U.S.P. Reference Std. ACTH (i.v.).

that reversals formed the consistent pattern in cats subjected to single, then combined, stimulation would indicate that if modulation does occur from remote areas of the central nervous system, it must act in a consistent way.

E. *Time course of corticosteroid alterations.* Significant alterations in corticosteroid levels were observed as early as 15 minutes after stimulation in some animals: recovery toward pre-stimulation levels might begin ½ to 1 hour after stimulation. In other instances, alteration in corticosteroid output achieved significant proportions only 1 to 2 hours after a stimulation. Thus both rapid and slow time courses of corticosteroid change were found,

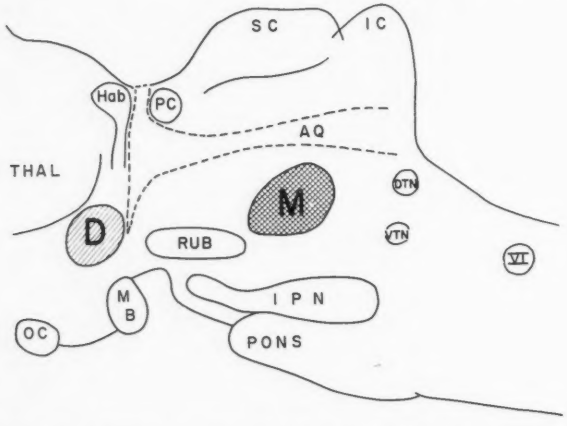

FIG. 1. Parasagittal reconstruction of cat brain stem. Stimulated sites in cross-hatched areas *D* in posterior diencephalon and *M* in midbrain. Abbreviations: AQ: cerebral aqueduct; DTN: dorsal tegmental nucleus; IC: inferior colliculus; IPN: interpeduncular nucleus; Hab: habenular nucleus; MB: mammillary body; OC: optic chiasm; PC: posterior commissure; RUB: nucleus ruber; SC: superior colliculus; THAL: Thalamus; VTN: ventral tegmental nucleus; VI: abducens nucleus.

even within the limitations of only two neuronal areas tested. This would suggest that ACTH release may be regulated by (facilitatory and inhibitory) components of more than one neuronal system.

F. *Histological location of stimulated sites.* Stimulated sites are shown in parasagittal reconstruction in Figure 1. Area *M*, in the posterior ventral midbrain tegmentum, is just antero-dorsal to the rostral pons, posterior to the red nucleus, ventro-lateral to the central grey and dorsomedial to the medial lemniscus. Area *D*, in the posterior diencephalon, includes field H_1 of Forel, the medial zona incerta, the subthalamic region, and the posterior hypothalamus dorsal or just anterior to the mammillary bodies. Histological examination of sites in area D failed to reveal any consistent difference in location of sites evoking increased, decreased or control levels of corticosteroid release.

G. *Independence of corticosteroid alteration from other physiological variables.* Although blood pressure rises of +10 to +30 mm. Hg were noted in 11 cats following stimulation in midbrain or diencephalon, only two were coincident with increased release of adrenal corticosteroids (cats 24 and 32, cf. Table 4). Blood pressures below 50 mm. Hg were correlated with decrease in corticosteroid release and deterioration in ECG, thus, as noted in *Materials and Methods*, all data has been eliminated for cats where blood pressure fell below 60 mm. Hg.

The adrenal blood flows on a five minute basis in the experimental series did not vary significantly from those noted for non-stimulated cats (range 2.1–4.3 ml.). In those cats whose blood flow increased with blood pressure no correlation could be made with either increased or decreased recovery levels of corticosteroids.

Single stimulation of the midbrain or diencephalon resulted in pupillary dilatation in 12 cats. No correlation could be made between presence or absence of pupillary dilatation with increases, decreases or normal release levels of corticosteroids, or with blood pressure changes.

In 12 cats during single diencephalic or midbrain stimulation the eyes moved to a conjugate contralateral position, with or without torsion (8); 10 of these cats also had pupillary dilatation during stimulation. In the remaining 12 cats stimulation did not affect spontaneous movements of the eyes. Evoked eye movements or their absence could not be correlated with changes in corticosteroid release.

Cortical arousal was observed in the ECG of 7 cats following stimulation of either single midbrain sites (Table 2, cats 14, 37) or combined midbrain-diencephalic sites (Table 4, cats 30, 32, 34, 36, 40). However, no correlation between this parameter and corticosteroid release was apparent.

The alterations of corticosteroid release observed in the present study would appear to be the specific effect of stimulation of neurons associated with the regulation of ACTH release. While alterations in other physiological variables were associated with many stimulations, none of these effects could be correlated with either the direction or the magnitude of alterations in adrenal effluent corticosteroids.

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EFFECT OF CHANGES IN PARATHYROID ACTIVITY ON BONE METABOLISM *IN VITRO*

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ABSTRACT

The effects of alterations in parathyroid activity *in vivo* on the mineral equilibrium between surviving bone and its bathing fluid have been examined *in vitro*. Weanling rats treated with parathyroid extract (PTE) or parathyroidectomized (PTX) during the week prior to sacrifice were compared with untreated controls. Thin fragments of freshly excised skull were incubated in bicarbonate buffer under 95%O_2, 5%CO_2. After 8 hours of incubation the mean calcium concentrations in buffer were $5.2 \pm .1$ mg.% for PTE skulls, $3.8 \pm .1$ for control skulls and $3.2 \pm .1$ for PTX skulls. The phosphorus concentrations were $4.2 \pm .1$, $3.8 \pm .1$ and $5.1 \pm .1$ mg.% respectively. Buffer citrate, lactate and pH did not differ significantly in the three treatment groups. The effects of prior treatment on buffer calcium concentration were similar after anaerobic incubation. Incubation of heat-inactivated bone resulted in a smaller final buffer calcium concentration. Addition of fluoroacetate to the buffer enhanced citric acid accumulation but buffer calcium concentration did not increase. Addition of iodoacetate inhibited lactate production and caused a marked decrease in buffer calcium concentration. These data indicate that the mineral equilibrium between surviving bone and its bathing fluid depends on metabolic activity and can be modified by prior changes in parathyroid function. Increased citrate production does not appear to be the mechanism of parathyroid hormone action. Glycolysis is active in bone tissue and appears to be required for calcium mobilization.

THE observations that parathyroid explants can lead to local bone resorption both *in vivo* (p. 2) and *in vitro* (3) and that removal of the parathyroid glands leads to a rapid fall in serum calcium (4, 5) have led to the hypothesis that the parathyroid glands maintain serum calcium by a direct effect on bone resorption (6). It has been assumed that the dynamic equilibrium between living bone and its bathing fluid must be maintained by cellular activity, since inert bone mineral cannot maintain the calcium and phosphorus concentration of the plasma by simple solubility (7). The

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present study demonstrates that the equilibrium between surviving bone and its bathing medium *in vitro* does depend upon metabolic activity and that this equilibrium can be altered by prior parathyroidectomy or administration of parathyroid extract. Possible effects on this equilibrium of organic acid accumulation, exchangeable bone calcium and conditions of incubation have been explored.

METHODS

Male albino rats (Holtzman farms) 25 to 35 days old were divided into control, parathyroid-extract treated (PTE) and parathyroidectomized (PTX) groups. The PTE rats were generally given 50 units of commercial parathyroid extract (Eli Lilly & Co.) by

FIG. 1. Appearance of weanling rat skull before and after slicing for incubation.

subcutaneous injection daily for three days and then 100 units daily for the next two days prior to sacrifice. The effect of other dose schedules was studied in a few experiments. Parathyroidectomy was accomplished under ether anesthesia 5 to 8 days prior to sacrifice with the aid of a dissecting microscope. At the beginning of each experiment, the rat was anesthetized with ether, bled by heart puncture and decapitated. The central flat portions of the parietal and frontal bones were removed (Fig. 1), the dura and periosteum dissected away and the skull then kept on filter paper moistened with iced saline. Each skull was cut into thin strips 1 to 2 mm. wide and approximately 0.4 mm. thick (Fig. 1). These strips were then placed in incubation flasks with 3 ml. of a Krebs-Henseleit bicarbonate buffer containing 100 mg.% of glucose. The composition of this buffer resembled rat extracellular fluid except in calcium and phosphorus concentration. The initial buffer calcium concentration was usually 3 to 5 mg.% and phosphorus concentration 3 to 4 mg.%, but these were systematically varied in selected experiments. Radioactive cal-

cium (Ca^{45}) and small amounts of penicillin and streptomycin were added to the buffer in some experiments. The skulls were then incubated at 37° C in a Dubnoff metabolic shaking incubator at 120 oscillations per minute. Incubations of 4, 8, and 24 hours were studied. The 8 hour period was selected for most studies since this period was required to achieve mineral equilibrium between bone and buffer. In each experiment aerobic incubation under 95%O_2, 5%CO_2 was carried out with skulls from at least three animals of each treatment group. In addition, the following conditions were compared with the standard aerobic incubation. 1. *Heat inactivation:* The skulls were heated to 80° C for 10 minutes in the buffer. Incubation was then continued as above. 2. *Anaerobic incubation:* The skull and buffer were placed in vessels capped with rubber and the contents equilibrated with 95%N_2, 5%CO_2 for 10 to 30 minutes at the beginning of incubation. 3. *Metabolic inhibitors:* Fluoroacetate and iodoacetate were added to the buffer prior to incubation, in final concentration of .002 M and .001 M respectively. Subsequently, incubation was carried out aerobically.

After varying intervals of incubation, samples of the buffer solution were obtained with a micro-syringe for stable and radioactive calcium determinations. When the experiment was terminated, the buffer solution was removed and precipitated with tungstic acid for citrate and glucose analysis or 10 gm.% trichloroacetic acid for lactate and phosphorus analysis. The buffer protein concentration after incubation was less than 0.1 gm.%. The bone was blotted dry and stored at −10° C for further analysis. In 2 experiments buffer pH was determined directly with an external electrode during incubation (Beckman, model GS, pH meter). In an experiment designed to determine CO_2 production, C^{14} labeled acetate was added to the initial buffer in tracer amounts and the skulls were incubated in this fluid for 4 hours. A center-well flask was used and the evolved CO_2 was collected as carbonate by adding KOH to this well and acidifying the buffer at the end of the experiment. The carbonate was then precipitated with barium sulphate and counted as barium carbonate. Weighed samples were counted in a micromil window counter and corrected for self-absorption. Ca^{45} was counted by evaporating .08 ml. samples of incubated buffer solution directly on planchettes and comparing the radioactivity with that of the initial buffer solution. Exchangeable calcium was then calculated as follows (8):

$$\text{exchangeable bone Ca (mg)} = \frac{\text{total counts added—counts remaining in buffer}}{\text{buffer specific activity (counts/mg. Ca)}}$$

Chemical Methods: The concentrations of calcium in plasma and buffer solution were determined by direct microtitration with ethylene-diamine-tetra-acetic acid in a photoelectric titrimeter (Evans Electroselenium, Ltd.) (9). Bone samples for Ca analysis were dissolved in 10 gm.% trichloroacetic acid and analyzed in the same manner except that the aliquots were mixed with equal volumes of .005M Na citrate to prevent precipitation when alkali was added. Phosphate was determined by the method of Fiske and Subba Row (10). Citrate in the buffer solution was measured by the method of Ettinger, Goldbaum and Smith (11) and in a trichloroacetic acid solution of bone by using the synthetic steps of this method with the thiosulfate color reaction described by Natelson, Pincus and Lugovoy (12). Lactate was determined by the method of Barker and Summersen as modified by Huckabee (13) and glucose with anthrone reagent (14). Non-collagenous nitrogen was determined by the method of Lowry, Gilligan & Katersky (15). Differences between groups means were tested by Student's t test. A P value of $<.02$ was selected as representing a significant difference.

RESULTS

Weanling rats in both treatment groups showed less rapid weight gain than controls (Table 1). Treatment with parathyroid extract for 5 days

TABLE 1. EFFECT OF PTE AND PTX ON BODY AND BONE WEIGHT AND BONE COMPOSITION

In this and subsequent tables, values given are mean and standard error, *n* is average number of animals in each treatment group.

	n	PTE	Control	PTX
Body wt. (gm.)	44	103 ±4**	121 ±4	99 ±2**
Skull wet wt. (mg.)	48	94 ±2*	106 ±3	102 ±4
Skull dry wt. (mg.)	39	47 ±1**	59 ±1	59 ±1
Total Bone				
Calcium (mg.)	46	9.3 ± .2**	11.1 ± .3	11.5 ± .3
Phosphorus (mg.)	36	5.7 ± .2**	7.0 ± .2	6.8 ± .2
Citrate (mg.)	26	.54 ± .03	.57 ± .03	.57 ± .03
Non-collagenous nitrogen (mg.)	15	.73 ± .05	.70 ± .05	.56 ± .05
Citrate/Ca (mg./gm.)	26	57 ±2*	49 ±2	47 ±1

* Significantly different from control values ($p < .01$).
** ($p < .001$).

resulted in a marked alteration in bone morphology with proliferation of connective tissue and lacunar reabsorption (Fig. 2). There was no detectable morphologic alteration of the bone after parathyroidectomy. The skulls of PTE rats showed a slight decrease in wet weight, but a greater decrease in dry weight and calcium content. The citric acid content of the skull was the same for the three treatment groups, but the citrate/calcium ratio was significantly increased in PTE bone. Neither non-collagenous nitrogen content of the skull, nor the ratio of non-collagenous nitrogen to calcium was significantly altered by treatment.

The plasma calcium concentration prior to sacrifice (Table 2) was increased by parathyroid-extract administration (PTE) and decreased by parathyroidectomy (PTX). When the skulls from these animals were incubated aerobically, the mean calcium concentrations in the buffer solution after 8 hours were 5.2 mg.% for buffer bathing PTE bone, 3.8 mg.% for buffer bathing control bone and 3.2 mg.% for buffer bathing PTX bone. These differences were highly significant and in individual experiments there was seldom overlap between final buffer calcium values for control and experimental flasks. Similar effects of treatment on the 8 hour buffer calcium concentration were observed regardless of the initial amount of calcium added to the buffer (Table 3). In separate experiments, omitting phosphorus from the initial buffer solution or increasing phosphorus con-

TABLE 2. EFFECT OF PTE AND PTX ON CALCIUM AND PHOSPHORUS CONCENTRATION (IN MG.%) IN PLASMA AND IN BUFFER AFTER 8 HOURS AEROBIC INCUBATION

	n	PTE	Control	PTX
Plasma				
Ca	101	11.4 ± .1*	9.6 ± .1	6.8 ± .1*
P	101	8.1 ± .2*	10.0 ± .3	14.2 ± .4*
Buffer				
Ca	43	5.2 ± .1*	3.8 ± .1	3.2 ± .1*
P	43	4.2 ± .1	3.8 ± .1	5.1 ± .1*

* Significantly different from control values ($p < .001$).

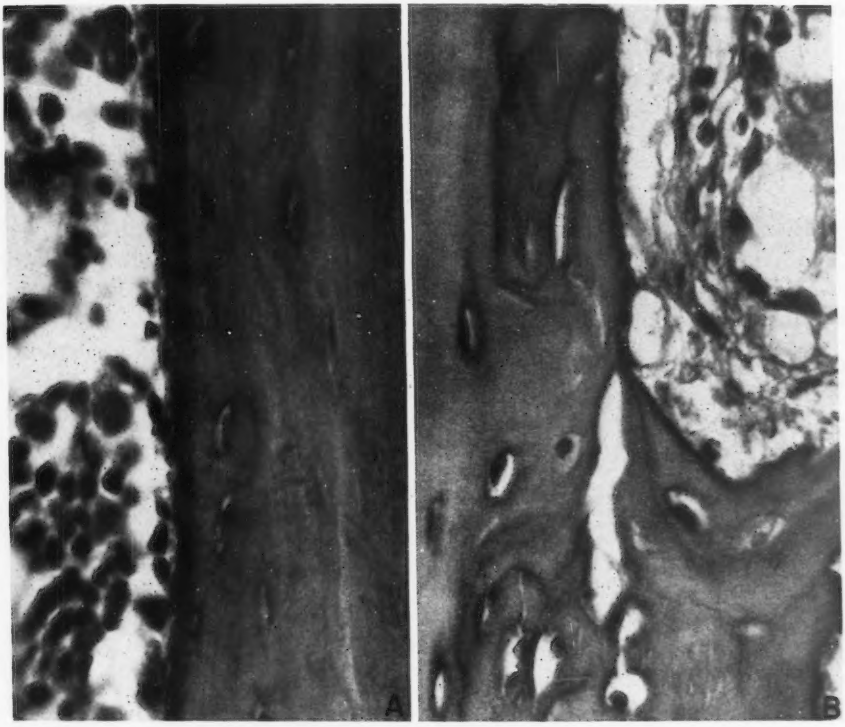

FIG. 2. Decalcified sections of skulls from control (A) and PTE (B) animals (×430). After PTE the bone showed lacunar resorption and proliferation of connective tissue in the marrow spaces, replacing hematopoietic tissue. Typical multinucleated osteoclasts were not seen.

centration to 7.5 mg.% had little effect on calcium mobilization. When the initial calcium concentration in the buffer was high the bone took up a small amount of calcium, when the initial concentration was low the buffer calcium content increased progressively during 8 hours of aerobic incubation (Fig. 3). Twenty-four hours of incubation of PTE bone gave no further increase.

TABLE 3. EFFECT OF VARYING INITIAL BUFFER Ca CONCENTRATION ON FINAL Ca CONCENTRATION AFTER 8 HOURS AEROBIC INCUBATION

Initial Ca, mg.%	*n*	8 hour Ca, mg.%		
		PTE	Control	PTX
6.2	10	5.7 ± .2	4.0 ± .1	3.3 ± .2
4.7	8	5.2 ± .2	3.8 ± .2	3.2 ± .2
3.3	6	5.3 ± .2	3.7 ± .1	3.1 ± .1
1.7	6	5.8 ± .3	3.3 ± .2	2.8 ± .2

When the dose of parathyroid extract was decreased from 350 units over 5 days to 150 units over 3 days, the final calcium concentration in buffer incubated with PTE skulls was significantly higher than for controls. Incubation of skull from animals killed one day after 50 units of parathyroid extract or one day after parathyroidectomy did not change final buffer calcium concentration when compared with controls.

Plasma phosphorus concentration decreased in PTE animals and increased in PTX animals. After incubation the final buffer phosphorus concentration was not significantly different for PTE and control bone, but was higher in the solution bathing PTX bone. The calcium phosphate ion product in weanling rat plasma was very high and was the same for PTE, PTX and control animals. The product was much lower in buffer than in

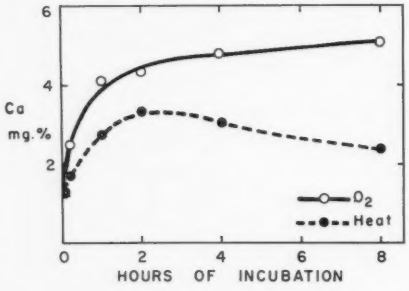

FIG. 3. Change in buffer calcium concentration during incubation of PTE bone. Calcium concentration increased progressively during aerobic incubation of fresh bone, but showed only a transient increase with heat inactivated bone. Each point represents the mean value for three vessels.

blood but the values for buffer incubated with PTE bone were greater than the values for buffer incubated with control and PTX bone.

The effects of changing the conditions of incubation on mineral equilibrium, glucose utilization and organic acid production are summarized in Table 4. The differences in buffer calcium concentration which developed during aerobic incubation were not associated with significant differences in glucose, lactate, citrate or hydrogen ion concentration when comparing buffer solutions containing PTE, control or PTX bone. When PTE, control and PTX bone were incubated anaerobically, their effect on buffer calcium concentration was similar to that observed after aerobic incubation. However, the mean buffer calcium concentration after anaerobic incubation of PTE was lower than the value for aerobic incubation. The decrease in buffer glucose concentration observed with aerobic incubation was not as great after anaerobic incubation. The average value for buffer lactate concentration was also lower after anaerobic incubation and this difference was significant for all three treatment groups. Although these mean values indicate some decrease in calcium mobilization and lactate production under anaerobic conditions, these changes were not observed in every experiment. As shown in Figure 4, there appeared to be a correlation between the effect of anaerobic incubation on lactate and on calcium content of the final buffer

TABLE 4. BUFFER COMPOSITION AFTER 8 HOURS INCUBATION OF PTE, CONTROL AND PTX SKULLS UNDER AEROBIC (O_2) OR ANAEROTIC (N_2) CONDITIONS AND AFTER HEAT INACTIVATION

		n	PTE	Control	PTX
Calcium (mg.%)	O_2	19	5.4 ± .1	3.9 ± .1	3.0 ± .1
	N_2	19	4.8 ± .2*	3.7 ± .2	2.8 ± .1
	Heat	9	3.2 ± .2*	2.0 ± .2*	2.2 ± .1*
Phosphorus (mg.%)	O_2	19	4.3 ± .3	4.1 ± .2	5.2 ± .3
	N_2	19	4.1 ± .3	3.9 ± .2	5.0 ± .3
	Heat	9	3.8 ± .2	3.6 ± .2	4.3 ± .3
Δ Glucose† (mg.%)	O_2	11	−37 ±6	−27 ±4	−18 ±2
	N_2	9	− 9 ±3*	−16 ±4	−12 ±5
	Heat	7	0*	0*	0*
Lactate (mg.%)	O_2	11	22 ±2	18 ±2	19 ±2
	N_2	9	14 ±1*	11 ±1*	14 ±1*
	Heat	3	0*	2*	4*
Citrate (mg.%)	O_2	8	1.3 ± .2	1.1 ± .1	1.3 ± .2
	N_2	3	1.6	1.7	1.4
	Heat	3	2.2	2.1	1.9
pH	O_2	6	7.26 ± .04	7.31 ± .03	7.33 ± .03
	N_2	6	7.24 ± .03	7.31 ± .04	7.31 ± .03

* Significantly different from values obtained with aerobic incubation ($p < .02$).
† Difference in buffer glucose concentration between buffer solutions incubated with and without bone.

solution. Anaerobic incubation did not alter final buffer pH or inhibit the release of citrate into the buffer.

Heating the bone tissue for ten minutes at 80° C before incubation resulted in a marked decrease in final buffer calcium concentration. The difference in buffer calcium concentration between control and PTX bone was abolished. After heat inactivation, PTE bone still released more calcium to the buffer than did control bone. However, this release was most

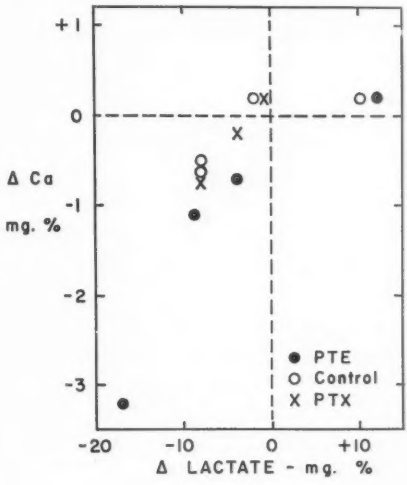

FIG. 4. Effect of anaerobic incubation on buffer calcium and lactate concentration. Each point represents the mean value for the difference between aerobically and anaerobically incubated vessels in the same experiment.

marked early in incubation and subsequently the bone took up calcium again (Fig. 3). Heat inactivation abolished glucose utilization and lactic acid production but did not inhibit citrate release.

Since tissue damage may have occurred during prolonged incubation, the metabolic changes during the first 4 hours were assessed in a separate group of experiments. After aerobic incubation of PTE skulls for 4 hours, the buffer calcium concentration was 3.1 ± .1 mg.%, the lactate concentration was 10.0 ± .6 mg.% and decrease in glucose concentration was 23 ± 3 mg.%. After anaerobic incubation these values were 3.4 ± .2 mg.%, 12 ± 1.4 mg.% and 27 ± 4 mg.% respectively. This similarity between the effects of aerobic and anaerobic incubation was also observed with control and PTX skulls. On the other hand, there was a striking reduction in the activity of the citric acid cycle. When a tracer dose of radioactive acetate was added to the

TABLE 5. THE FRACTION OF BONE Ca EXCHANGING WITH BUFFER AFTER 8 HOURS INCUBATION OF PTE, CONTROL AND PTX SKULLS UNDER AEROBIC (O_2) AND ANAEROBIC (N_2) CONDITIONS AND AFTER HEAT INACTIVATION

		n	PTE	Control	PTX
Exchangeable Ca (% of Bone Ca)	O_2	19	5.5 ± .3	5.6 ± .2	4.0 ± .1*
	N_2	10	6.3 ± .4	5.0 ± .4	4.2 ± .1
	Heat	7	5.8 ± .6	5.6 ± .5	4.3 ± .3

* Significantly different from control values ($p < .001$).

buffer, 8 to 10% of the radioactivity was recovered in 4 hours as $C^{14}O_2$ under aerobic conditions. However, under anaerobic conditions, no significant amount of $C^{14}O_2$ was recovered. There was no difference in the amount of $C^{14}O_2$ produced under aerobic conditions by PTE, control or PTX skulls.

The fraction of bone calcium which had exchanged with buffer after 8 hours of incubation was not altered by PTE treatment (Table 5). The exchangeable calcium was significantly decreased in PTX bone. Anaerobically incubated and heat inactivated bone gave similar results. After one hour of incubation the mean values for exchangeable calcium were 1.1% for PTE bone, 1.2% for control bone and 1.0% for PTX bone.

Fluoroacetate inhibits citric acid oxidation and increases citrate concentration in many tissues (16). When .002 M fluoroacetate was added to the buffer during aerobic incubation, citrate concentration increased (Table 6) but this increase could account for the chelation of only 5 to 10% of buffer calcium. Buffer calcium concentrations were decreased in the control and PTX groups but the differences between control and treatment groups were proportionate to those obtained without fluoroacetate. One and 4 hour samples also showed some decrease in buffer calcium in the presence of fluoroacetate. The addition of .001 M iodoacetate inhibited glycolysis and impaired lactate production. There was a decrease in calcium concentration in the buffer, as great as that observed with heat inactivated skulls

TABLE 6. EFFECT OF FLUOROACETATE (FAc) ON BUFFER Ca AND CITRATE CONCENTRATION AFTER 8 HOURS AEROBIC INCUBATION OF PTE, CONTROL AND PTX SKULLS

	n	PTE	Control	PTX
Calcium—without inhibitor	5	4.6 ± .1	3.3 ± .1	2.4 ± .1
with .002 M FAc	5	4.1 ± .2	2.5 ± .1*	1.9 ± .1*
Citrate —without inhibitor	5	1.1 ± .1	0.9 ± .1	1.0 ± .1
with .002 FAc	5	1.9 ± .1*	1.4 ± .1*	1.3 ± .1

* Significantly different from values without inhibitor ($p < .02$).

(Table 7). Nevertheless, the differences in buffer Ca concentration between control and treatment groups persisted ($P < .01$).

DISCUSSION

The present study demonstrates that surviving bone can maintain a higher calcium and phosphorus concentration in its bathing fluid *in vitro* than bone tissue inactivated by heat or by a metabolic inhibitor. The data thus provide direct confirmation of the hypothesis that metabolic activity maintains the mineral equilibrium between bone and its bathing fluid. A similar result has recently been reported for human bone by Bassett and Nordin (17). The present study further demonstrates that bone-buffer equilibrium can be altered by changes in parathyroid activity induced prior to sacrifice. This alteration appears to depend at least in part on metabolic activity, but can occur in the absence of aerobic metabolism.

The excised skull probably remains metabolically active in a simple buffer solution containing glucose for 24 hours. Nevertheless, calcium and phosphorus ion concentrations are not as high as those found *in vivo*. A better approximation of *in vivo* calcium and phosphorus concentrations has been obtained in tissue culture (18) using a more complete medium. It is probable that damage to cells is occurring during the incubation period. Such damage might be greater under anaerobic conditions and could account for the partial inhibition of calcium mobilization observed after 8 hours of anaerobic incubation in some experiments.

Heat inactivation decreased calcium mobilization and abolished the difference between control and PTX bone. There was still a transient release of more calcium from PTE bone. Possibly prolonged PTE administra-

TABLE 7. EFFECT OF IODOACETATE (IAc) ON BUFFER Ca AND LACTATE CONCENTRATIONS AFTER 8 HOURS INCUBATION OF PTE, CONTROL AND PTX SKULLS

	n	PTE	Control	PTX
Calcium—without inhibitor	8	4.9 ± .2	3.7 ± .1	2.8 ± .1
with .001 M IAc	5	3.0 ± .1*	2.5 ± .1*	2.1 ± .1*
Lactate —without inhibitor	8	24 ±1	21 ±2	17 ±1
with .001 M IAc	5	1 ±1*	1 ±1*	1 ± *

* Significantly different from values without inhibitor ($p < .001$).

tion caused an alteration in bone crystals or changed their relationship to matrix. However, the values for exchangeable bone calcium obtained by addition of Ca^{45} *in vitro* could not be correlated with the ability of the bone to mobilize calcium. The decrease in exchangeable calcium observed after parathyroidectomy confirms previous data obtained in living animals (8). This effect has been ascribed to a decreased rate of new bone formation.

The administration of parathyroid extract for several days prior to sacrifice was required to obtain maximal effects on calcium mobilization *in vitro*. In attempts to demonstrate a direct *in vitro* effect of parathyroid hormone, varying amounts of both commercial and specially purified parathyroid extract have been added to buffer containing control or PTX bone and incubated for 8 to 24 hours. In 2 experiments a small but significant increase in buffer calcium concentration was obtained at 8 hours with the addition of 12 units of parathyroid extract, but in 6 other experiments no change occurred. The reason for this discrepancy is not known.

Neuman (7) has suggested that a local increase in citric acid concentration might mobilize calcium from bone mineral. Data on buffer citrate concentrations in the present study do not support this concept. The citrate/calcium ratio in bone was increased after PTE administration, confirming the previous finding of Dickens (19). However, this need not indicate increased citrate production. Newly formed bone crystals have a higher citrate/calcium ratio (20) and PTE treatment could increase the proportion of such crystals in bone by active resorption of older crystals and stimulation of new bone formation. Incubation of bone with fluoroacetate increased buffer citrate but actually decreased calcium mobilization. The results of such prolonged incubation with a metabolic inhibitor are difficult to interpret. Both citrate release and decreased calcium mobilization may have been due to cell damage. These effects were most marked with bone from PTE treated animals, possibly because this bone was initially more cellular and more metabolically active. Nevertheless, this observation and the failure of anaerobic incubation to inhibit calcium mobilization argue against the citric acid theory.

It is likely that glycolysis plays an important role in calcium mobilization by bone. Bone tissue produces large amounts of lactic acid from glucose even under aerobic conditions (21, 22). Iodoacetate inhibited glycolysis and lowered buffer calcium concentration to a degree comparable to that achieved with heat inactivation of the bone. Paradoxically, lactate production sometimes decreased under anaerobic conditions in the present study. This decrease was also associated with lower buffer calcium concentrations. During less prolonged anaerobic incubation, lactate production by bone may be increased (22), but a striking Pasteur effect of anaerobiosis on glucose utilization and lactate production has not been found in bone.

If the acid products of glycolysis were released from cells at the bone

surface, the lower pH could result in calcium mobilization (23). It is difficult to conceive that such a phenomenon operating alone could raise calcium concentration in the bathing medium without causing a greater decrease in buffer pH than that observed in the present study. An alternative hypothesis is that glycolysis is required for the active cellular transport of calcium from the bone to the bathing medium. Possibly both mechanisms operate synergistically. In any case, there must be an active cell layer interposed between bone and the circulating extracellular fluid. Interruption of this layer by slicing the tissue could provide another explanation for the failure of buffer calcium ion concentration to reach the values found in the plasma prior to sacrifice. Whatever the function of glycolytic activity in bone cells, the effect of parathyroid hormone could be to stimulate this activity or to increase the number of active cells. The latter phenomenon is readily apparent in microscopic sections of PTE bone and could account for the relatively slow action of parathyroid extracts in the present study. Such an effect would be consistent with the recent observation that prior administration of parathyroid hormone may increase aerobic glycolysis in the presence of glucose in short term incubations (24).

Acknowledgment

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THE ARREST OF PROLONGED ESTRUS IN "MIDDLE-AGED" RATS BY PINEAL GLAND EXTRACT[1,2]

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ABSTRACT

Protein-free bovine pineal extract altered the persistent state of estrus of female rats of post-reproductive age, but had no influence on the regularity of the estrous cycle in adult animals in the doses administered. Ovarian weights were significantly lower in pineal treated animals. Histological examination showed more interstitial tissue, larger though fewer corpora lutea, fewer follicles, and a greater abundance of wheel cells in the ovaries of "middle-aged" treated rat. A possible relationship between the pineal and pituitary gonadotrophic hormone is suggested.

INTRODUCTION

THE possible relation between the pineal gland and reproductive systems has been a subject of study for many years. A review of the literature on this topic to 1954 has been published in a monograph by Kitay and Altschule (1). A significant advance since that time has been the demonstration (2, 3, 4) that pinealectomy in immature female rats is followed by increases in ovarian weight but not by concurrent changes in uterine weights. In pre-pubertal animals, premature opening of the vagina was observed (1). The administration of pineal gland extracts caused a marked decrease in ovarian weights of both intact and pinealectomized animals, and produced histologic evidence of retarded ovarian maturation in females (1, 3). A study of this dose-response has been carried out by Wurtman, Altschule and Holmgren (5).

In the past year this problem has been pursued further, by means of a study of the effect of pineal extract on ovarian function of adult and older rats as shown by histological changes in vaginal smears. It was found that in animals capable of reproduction the estrous cycle proceeded normally even after large doses of pineal extract had been given. However, in older

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rats, those of about 18 months of age in which a persistent state of estrous prevails (6), a state of anestrous could be brought about by the administration of pineal extract. Cessation of the treatment permitted a return to the previous state. This paper describes these experiments and presents a general discussion of the possible relationship of these and other findings to the pattern of gonadotrophin secretion by the pituitary.

MATERIAL AND METHODS

Female rats of the Charles River CD strain were used in this study. The 18-month-old animals were obtained from the Laboratory of Vitamin Technology, Chicago, or from stocks kept in our animal quarters.

The life span of the female rat can conveniently be divided into four stages (6): 1) the first 52–56 days, the period of immaturity; 2) 54 days to the end of the first year, the period of reproductive capability; 3) the second year, the period of post-reproductivity, or "middle-age" which is accompanied by a persistent state of estrus; 4) the third and last year, where anestrous prevails. The animals in these categories will be differentially referred to in this paper as immature rats, adult rats and "middle-aged" rats respectively for the periods listed. Rats in categories 2 and 3 were used in this study.

The preparation of protein-free bovine pineal gland extract has been described previously (7). Administration of this material or of saline solution was by intraperitoneal injection. Vaginal smears were taken once each day and stained according to the technique of Papanicolaou and Shorr (8).

OBSERVATIONS

Experiment 1: Twelve 54 day old female rats were sorted into two equal groups; each rat was injected daily as follows:

Day	*Group I*	*Group II*
1– 18	No injection	No injection
19– 31	1 ml. saline	1 ml. pineal extract
32– 42	2 ml. saline	2 ml. pineal extract
89– 97	No injection	No injection
98–108	1 ml. saline	1 ml. pineal extract
109–119	2 ml. saline	2 ml. pineal extract

During the first 18 days after vaginal opening in these rats 4- to 5-day estrous cycles were observed. Thereafter daily injections of 1 ml. of either saline solution or pineal extract for 12 days brought about no change in duration of the cycles. The increase of the dosage to 2 ml. per day still did not bring about any alteration of the normal cycle. The possibility that the failure of the pineal extract to elicit any effects might be due to the age of these animals was considered, and the same animals were subjected to a second schedule of treatment 2 months later. Results identical to those in the first series were obtained.

Experiment 2: 6 female rats, 18 months old, were sorted into two equal groups and each rat injected daily as follows:

Day	*Group I*	*Group II*
1–10	1 ml. saline	1 ml. saline
11–20	1 ml. pineal extract	1 ml. pineal extract
21–35	0.5 ml. saline	0.5 ml. pineal extract

Experiment 3: 16 female rats, 18 months old, were sorted into two equal groups and each rat injected daily as follows:

Day	*Group I*	*Group II*
1–10	1 ml. saline	1 ml. pineal extract
11–31	No injection	No injection
32–42	1 ml. saline	1 ml. pineal extract

Experiments 2 and 3 were carried out with rats of post-reproductive age. During the period of saline treatment, the vaginal smears of all rats showed mostly cornified non-nucleated cells, as expected, indicating a constant state of estrous. By the second day after the initiation of pineal extract (1 ml.) injections, 9 out of 11 animals no longer showed these cells, but a predominance of leucocytes. This state of anestrous prevailed through the period of pineal extract administration, ceasing only on about the second day after these injections were withdrawn or saline substituted for them. In the experimental group continued on 0.5 ml. of pineal extract daily, all animals remained in anestrous, indicating that this dose of extract was still adequate for the arrest of estrous. Typical data for a single experimental group of rats are illustrated in Figure 1.

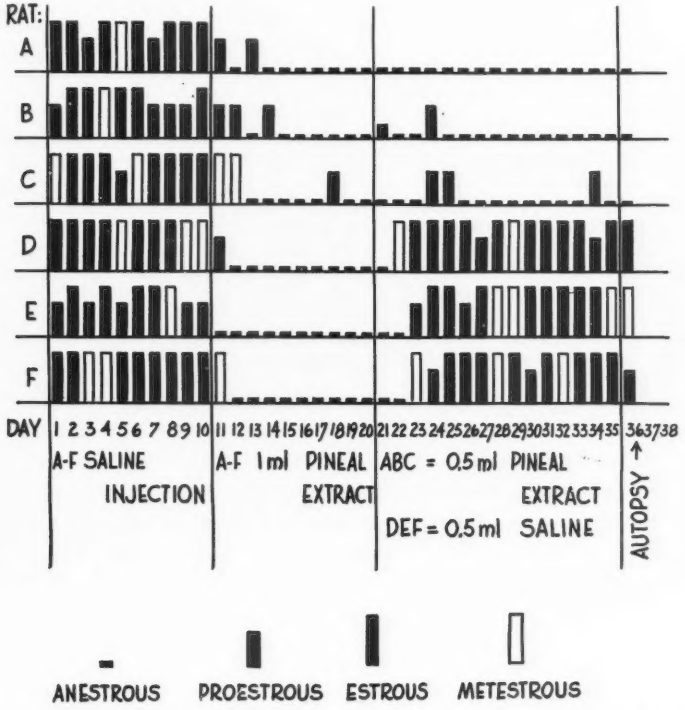

FIG. 1. Daily vaginal examinations of old rats treated with pineal extract.

One day after the termination of the injection schedule, the rats were sacrificed and organ weights noted:

	Mean organ weights: mg. ±S.D./100 gm. body wt.			
	Ovary	*Uterus, wet wt.*	*Uterus, dry wt.*	*Adrenals*
Pineal extract group	30.0±0.923	147±39.7	29.8±3.96	11.2±0.711
Saline group	40.4±2.44	202±33.6	39.0±4.30	18.4±0.927

The effect of pineal extract on ovarian weight in these "middle-aged" rats is dramatic and is identical to the findings of previous investigators (3, 5) on immature rats. There appears also to be some effect on uterine weights. No decrease in size of this organ was observed in work with rats of reproductive age (4). Adrenal weights were also tabulated; these data are in keeping with those documented by Wurtman *et al.* (5). Histological examination of the ovaries of the animals in the above experimental groups was also carried out. In the control group, i.e. those receiving only saline in periods of injection, there appeared larger numbers of follicles in all stages of development, and less interstitial tissue. Corpora lutea were present in both ovaries although these were considerably larger though less in number in those of the treated group. The so-called wheel cells (9) were more numerous in the ovarian interstitial tissue of animals given pineal extract than in that of untreated animals.

DISCUSSION

Various investigators (10, 11, 12) have described the occurrence of persistent estrous in adult rats after constant exposure to light. The animals used in the present study were kept in a windowless room with artificial illumination which was turned on for approximately three hours per day. No instance of persistent estrous was noted in our adult rats under these conditions. Gronroos and Kauppila (13) have found that rats under extreme stress go into a state of anestrous. It is unlikely that the injections of pineal extract in our experiments constituted any significant degree of stress; the results observed being due to the contents of the pineal extract itself.

It has been recognized that the administration of gonadotrophins has the following effects in rats: Premature opening of the vagina in pre-pubertal animals (14), hypertrophy of the ovaries (15) and rejuvenation of the estrous cycle in animals of post-reproductive age (16). The administration of pineal gland extract provokes responses directly opposite to these (1, 3). Moszkowska (17) has recently reported experiments where the grafting of the anterior pituitary of an adult male rat to the ovary of an immature female resulted in precocious puberty. When the pineal gland was also grafted, puberty did not appear. The possibility that the pineal in some way controls the secretion or production of the gonadotrophic hormones suggests itself.

It was interesting to find in the present study that the pineal gland extract in doses which had been shown to cause a marked decrease in weight of the ovaries of adult rats, appeared to have no effect on the function of the ovary insofar as the estrous cycle was concerned It is possible that larger doses of pineal extract than those used might have evoked some change in adult rats. It was not determined whether this dose of extract could influence fertilization or pregnancy. (The scarcity of this material precluded both these experiments.) "Middle-aged" rats, in which persistent estrous has been attributed by Bloch and Flury (6) to the continued but *diminished* secretion of gonadotrophins by the pituitary were affected by pineal extract. It seems unlikely that the pineal extract is acting directly on the ovary since hypophysectomized rats have not elicited the ovarian responses observed in intact animals (4). Thus in terms of a possible pineal-anterior pituitary interplay, the arrest of estrous in the "middle-aged" animals by low doses of pineal extract suggests that the level of gonadotrophins in these animals may be critical, in contrast to adult rats where doses of as large as four times that which caused a maximal response in old rats had no influence. The interruption of estrous with pineal extract could be due to inhibition of gonadotrophin elaboration.

The histology of the ovaries of rats has had thorough study (15, 18, 19). Definitive changes noted with advancing age are the decrease in number of follicles, the increase in size of corpora lutea and an increase in the number of wheel cells. In the present study, the control group showed distinct evidence of continued, though diminished gonadotrophin response as manifested by the presence of normal, atretic and cystic follicles. When pineal extract was given the ovarian picture changed in the direction of gonadotrophin withdrawal, reflecting completely the pattern noted above with rats of advancing age.

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THE LOCALIZATION OF ACID PHOSPHATASE ACTIVITY IN THE RAT PITUITARY AND THYROID GLANDS, AND ITS RELATION TO SECRETORY ACTIVITY[1]

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ABSTRACT

The histochemical distribution and quantitative chemical and histochemical changes in acid phosphatase activity were studied in rat pituitary and thyroid glands in various phases of secretory activity.

The histochemically determined enzyme activity paralleled hormonal secretion. This could be confirmed chemically for the thyroid.

The acid phosphatase activity appeared to be localized in the Golgi apparatus in these organs.

ACID phosphatase is widely distributed in many seemingly unrelated organs and cells. High activity of this enzyme has been described in endocrine organs such as pituitary (1), thyroid (2) and pancreatic islets (3). The description of its distribution in pituitary and thyroid has been sketchy, with considerable artefactual staining because of the use of older histochemical methods. In an attempt to define more clearly the relation of acid phosphatase activity to hormone production or secretion, the histochemical distribution and changes in acid phosphatase activity were studied in these glands in various phases of hormonal activity. A new azo-dye method for the demonstration of acid phosphatase was used (4).

MATERIAL AND METHODS

Carworth Farms CFE (Sprague-Dawley descendant), male, albino rats of approximately 120 gm. were kept 5 in a cage and had free access to water and food (Rockland Rat Pellets). One group of these animals (20 rats) was kept at $4 \pm 1.5°$ C. Five of these rats were killed at 7 days and 5 at 21 days for histochemical studies. The remainder of these rats were killed at 21 days for chemical determination of acid phosphatase activity.

Another group of animals (30) was kept at room temperature ($23 \pm 1.5°$ C). Half of these animals were injected subcutaneously daily with 10 μg of DL-thyroxine (Nutritional Biochemicals Co.) per 100 gm. of body weight and killed after 28 days; 5 of these animals were studied histochemically and the remainder chemically. The 15 control animals, 7 of which received daily injections of the thyroxine solvent, were killed simultaneously with the experimental animals and similarly studied.

The animals were killed by exsanguination under ether anesthesia. For histochemical

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studies thyroids and pituitary glands were fixed for 24 hours in cold formol-calcium, pH 7.0. Frozen sections were stained by a modified azo-dye method (4) for acid phosphatase, and parallel sections with hematoxylin and eosin. Pituitary basophils were further characterized by the PAS reaction with and without acid phosphatase staining, and thionin counterstaining. The criteria of Halmi (5, 6) and Purves and Griesbach (7) (i.e. localization and shape of cell and nuclear contour) were utilized in differentiating thyrotrophic from gonadotrophic basophils. Aldehyde fuchsin staining of the frozen sections was unsuccessful.

For chemical analysis the thyroid and pituitary glands were dissected free of nonglandular tissue, weighed and quickly frozen. A 1% homogenate in distilled water was prepared using a Potter-Elvehjem homogenizer. The homogenates were frozen and thawed. The incubation mixture consisted of 0.1 ml. of homogenate, 0.5 ml. of Michaelis veronal-acetate buffer, pH 5.0, and 0.2 ml. of a 0.4% *p*-nitro-phenolphosphate solution in 0.001N HCl. After 30 minutes at 37.5° C the reaction was stopped by adding 1 ml. of 5% trichloracetic acid, and after adding alkali the optical densities were read on duplicate samples at 420 mμ using a Beckman DU Spectrophotometer (8, 9).

RESULTS

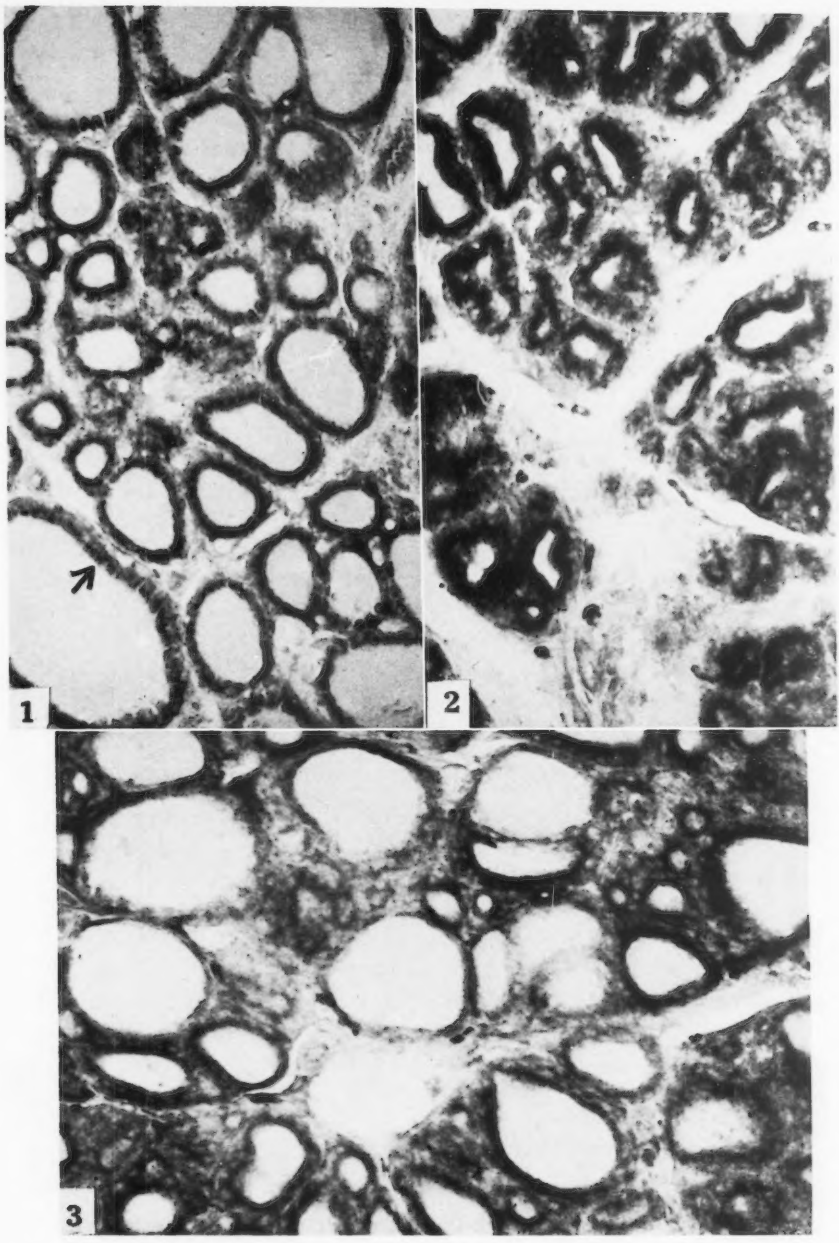
Histologic Observations

Thyroid. The control animals with or without buffer treatment showed normal thyroid structure consisting of follicles of variable size, filled uniformly with acidophilic colloid, and lined by cuboidal to slightly flattened cells with basal nuclei. Occasional acini were smaller and lined by low columnar epithelium. The acid phosphatase activity (Fig. 1) was localized in the cytoplasm supranuclearly in the form of fine and occasional coarse granules. Sometimes these deposits surrounded the nucleus as a crescent. There was considerable variation in the activity in individual acini. The columnar cells of the occasional smaller acinus contained more and coarser granules more concentrated in an oval supranuclear area.

Rats exposed to cold had diffusely hyperplastic glands. After 7 days exposure the thyroid follicles were almost uniformly smaller, lined by columnar cells, and contained little colloid which in some areas exhibited scalloped margins. These changes were more pronounced after 21 days. The acid phosphatase activity (Fig. 2) was more conspicuous and diffuse, and the dye deposit was coarser than in the control glands, though similarly localized. An oval supranuclear zone containing the bulk of the precipitate was poorly demarcated from the remainder of the cytoplasm which showed considerable activity.

After thyroxine treatment the thyroids were composed almost uniformly of large follicles with flattened to low cuboidal epithelium, and filled with eosinophilic colloid. The acid phosphatase activity (Fig. 3) was localized in the same area as in the control animals, but the intensity of staining was much diminished, and the granules much finer. No oval supranuclear concentration could be discerned except vaguely in the few smaller acini composed of columnar cells. The colloid showed no enzyme activity.

Pituitary. In the control rats the highest acid phosphatase activity (Fig.



All photomicrographs are of preparations stained for acid phosphatase without counterstaining.

FIG. 1. Thyroid of control animal. The acinar cells are cuboidal and stain with moderate intensity. Note oval supranuclear dye deposits (arrow). (200×)

FIG. 2. Thyroid of rat exposed to cold for 21 days. The acinar cells are columnar with markedly increased staining supranuclearly. The acini exhibit papillary infoldings and contain little colloid. (200×)

FIG. 3. Thyroid of rat treated with thyroxin for 28 days. Note light staining of flattened thyroid acinar cells. The acini contain abundant colloid. (200×)

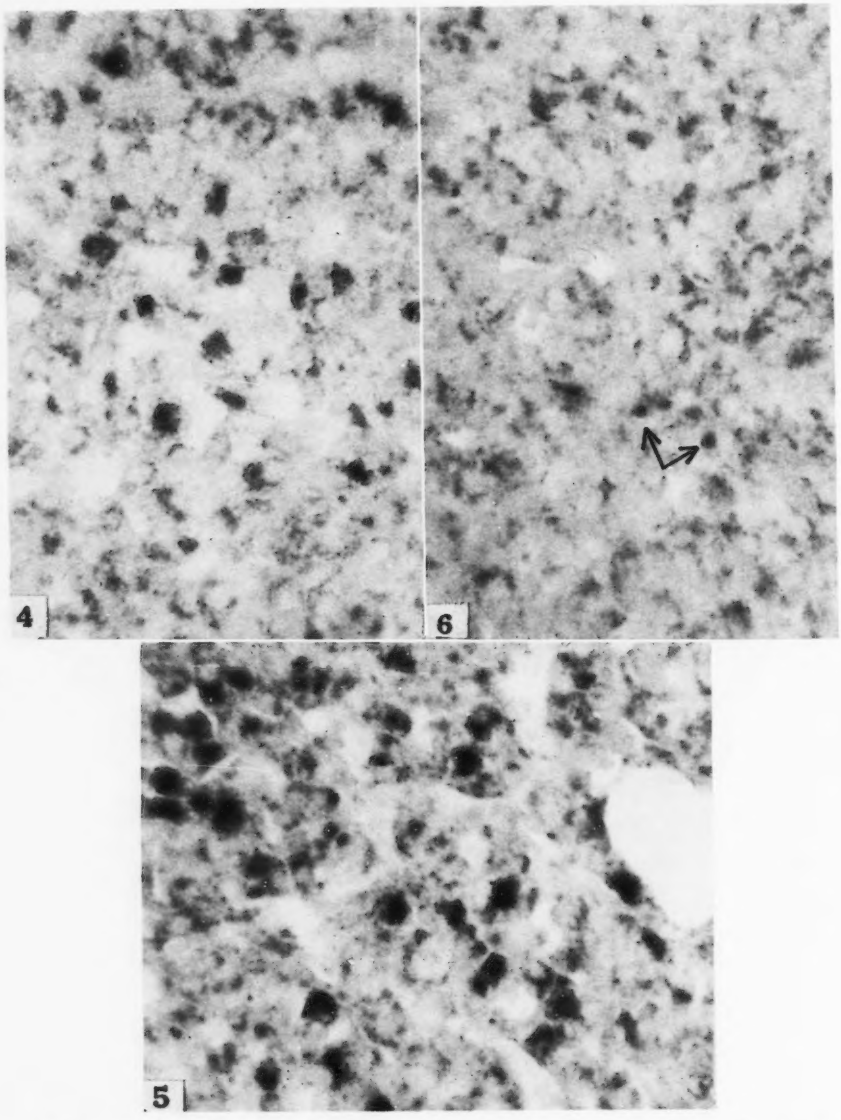


FIG. 4. Pituitary of control animal. Compare larger more dense oval collections of azo dye in thyrotrophs with figure 6. Semilunar areas of activity are seen in eosinophils. (460×)

FIG. 5. Pituitary of rat exposed to cold for 21 days. Compare larger more dense deposits of azo dye in thyrotrophs with figure 4. (460×)

FIG. 6. Pituitary of rat treated with thyroxin for 28 days. A small amount of azo dye is noted in thyrotrophs in the form of small oval collections (arrow) (460×)

4) was seen in the anterior lobe in round, well demarcated, paranuclear areas of the PAS positive (thyrotrophic and gonadotrophic) cells. These areas composed of moderately coarse granules appeared to be within a vesicular structure containing some PAS positive material, and were usually superimposed upon it. Basophils without this oval area of activity were rare. Many cells that were thought to be eosinophils revealed a finer, granular concentration of activity in a semilunar paranuclear zone. Both types of chromophil cells also contained a small amount of diffuse cytoplasmic activity. In the chromophobes, minimal diffuse cytoplasmic staining and occasionally similar, but smaller, less coarse, oval and semilunar deposits of activity were noted. Most cells of the pars intermedia revealed a fine sprinkling of cytoplasmic activity. In a few cells an oval, more dense, coarser, paranuclear concentration of activity of smaller dimension than in the anterior lobe was observed. It was frequently associated with PAS positive cells having pseudopod like cytoplasmic projections. A few large ill-defined foci of activity which in many instances corresponded to lakes of PAS positive material were noted in the posterior lobe. The pituicytes showed coarse granular deposits of activity. The colloid between the pars intermedia and anterior lobe had no enzyme activity. Large macrophages usually present in the colloid exhibited a high activity. Littoral cells occasionally revealed small clumps of activity.

After cold exposure the pituitary revealed an increase in size and number of thyrotrophs. Their paranuclear vesicular structure, thought to be the negative image of the Golgi apparatus, was larger, contained more PAS positive material, and their cytoplasm showed less intense PAS staining. These cells revealed an increased acid phosphatase activity (Fig. 5) and coarser granularity of the dye deposit mostly in the oval paranuclear structure, but also diffusely in the cytoplasm. Otherwise no difference from control animals was apparent.

After thyroxine treatment, the thyrotrophs revealed decreased size, acid phosphatase activity (Fig. 6) and PAS positivity of the oval, paranuclear vesicular structure. Their cytoplasm contained considerably less activity, and the precipitate was finer than in the controls. They were otherwise similar to the controls.

In both experimental groups many PAS positive cells showed a reaction similar to that in the control pituitaries. These cells had the appearance and distribution of gonadotrophs.

Chemical Determination (see Table 1)

The acid phosphatase activity of the thyroid expressed on a wet weight basis was more than twice that of the pituitary. Thyroxine administration resulted in a diminution and cold exposure in an increase of acid phosphatase activity in the thyroid. These procedures caused similar changes in the pituitary which however were not statistically significant. The discrepancy

TABLE 1

Treatment	No. of animals	Acid phosphatase activity,* thyroid†	P**	Acid phosphatase activity,* pituitary†	P**
None (control)	8	5.67 ±0.31		2.48 ±0.47	
Thyroxine	9	3.15 ±0.55	<0.001	2.21 ±0.32	>0.1
Cold	6	6.75 ±0.44	<0.01	3.02 ±0.43	<0.1

* In mM $PO_4^{\equiv}$ liberated per 60 min. per gm. of wet tissue.
** Probability of the significance of the difference from the control.
† With standard deviations.

between the histochemical picture and chemical determination in the pituitary may be explained by the relatively small numbers of cells involved.

DISCUSSION

The histochemical distribution of acid phosphatase activity in the pituitary and thyroid has previously been mentioned. Using a new modified azo-dye technique allowing more precise localization, high activity of this enzyme was noted in the pituitary and thyroid. The acid phosphatase activity of the thyroid acinar cells and pituitary thyrotrophs was characteristically altered by exposure to cold and twice the amount of thyroxine adequate as replacement therapy and sufficient to depress TSH secretion by the pituitary (13). Exposure to cold produces an increased secretion of TSH by the pituitary thyrotrophic cells with a concomitant hyperplasia of the thyroid gland, and increased thyroxine production (10–19). The enzyme activity shown histochemically in these cells was increased in states of increased hormonal output and conversely decreased in states of diminished output. This could be related either to the production or secretion of hormones. The mass and number of affected cells changed with the treatment, and this also was reflected in the quantitative data. Schreiber and Kmentova (20) presented chemical data showing statistically significant changes in pituitary and thyroid acid phosphatase activity in methylthiouracil induced thyroid hyperplasia and after thyroxine treatment. Their data revealed a lesser alteration in enzyme activity with treatment in the pituitary than the thyroid, but a greater magnitude of change than in our experiments. Changes in the acid phosphatase activity of thyrotrophs cannot be expected to significantly influence the enzyme activity of the pituitary determined chemically because thyrotrophs are not the only carrier of acid phosphatase activity in the pituitary and represents only a small percentage of its activity.

Acid phosphatase has been described in many organs and cells. Its function is unknown. The new lysosome concept of deDuve (21) seems to offer a plausible working hypothesis as to the role of this and other hydrolytic enzymes. According to deDuve (21) and Novikoff (22) these enzymes are found in an intracellular structure, the lysosome, surrounded by a double

membrane of lipo-protein character. The localization of acid phosphatase in the peribiliary zone of the hepatic cell and in the renal tubular cell suggests a role in excretory or secretory mechanisms. In addition to the lysosome, prominent deposits of acid phosphatase were found in the Golgi region in many organs and species (23, 24). Sharma (quoted by El Mofty (25)) pointed out that acid phosphatase granules seem to follow the fate of the Golgi apparatus and postulated that the Golgi apparatus might secrete this enzyme. El Mofty (25) demonstrated that the only organelle which shows acid phosphatase activity in one protozoan is the parabasal apparatus which under the electron microscope has the same composition as the Golgi "cords" of secretory cells (26). The Golgi zone is known to increase in size and complexity in actively secreting cells, and involute with diminished secretory activity (27, 28). It is considered a cellular center of synthesis. Secretion granules are first noted in this zone in the pituitary (29, 30). The observations seem to indicate that acid phosphatase may be confined to the Golgi zone and to the lysosomes; however, further studies are required to determine the exact relationship of acid phosphatase to these organelles. Located in these organelles, acid phosphatase may participate either in the production or the secretion of the hormone or in both of these processes.

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SEVERE KETOACIDOSIS IN THE ALLOXAN DIABETIC RAT[1]

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ABSTRACT

Treatment of non-ketotic alloxan diabetic rats for several weeks with daily injections of 4 to 6 units of protamine-zinc insulin resulted in marked weight gain, decreased glucosuria, and apparent restoration of normal health. When insulin was withdrawn, the animals developed abrupt weight loss, marked ketonemia, ketonuria, hyperglycemia, glucosuria, acidosis, Kussmaul respiration, coma, hypothermia, and death within 60–96 hours. Postmortem examination revealed severe fatty infiltration in the livers and kidneys. Gradual insulin withdrawal resulted in weight loss, transient ketonuria, and the slow return of the characteristic picture of severe non-ketotic alloxan diabetes. The severity of ketonemia was proportional to insulin dosage and to gain in body weight during therapy for 40 days with a range of doses from 0.5 to 4.0 units Protamine-zinc insulin daily.

MANY descriptions have been recorded of coma associated with ketonuria or ketonemia occurring within a few days after the injection of alloxan into rats. Recently Scow and co-workers (1, 2, 3) have described in detail the intense diabetic ketoacidosis which develops in rats after almost complete excision of the pancreas. Previous attempts to produce such a severe diabetes in rats by pancreatectomy were unsuccessful, apparently because of the large reserves of β cells of the species and the technical difficulties of pancreatectomy. Indeed, Foglia (4) had estimated that 95% pancreatectomy or more was necessary to produce permanent diabetes.

Although a short period of ketonuria, lasting about 7 days, has been noted following the injection of alloxan (5), it has been clearly demonstrated that alloxan also produces transient hepatic and renal impairment (6, 7). Azotemia may be severe for 1 to 3 weeks after alloxan administration, and initially the severity of the azotemia and of the diabetes run a parallel course (8). Thus coma and acidosis early after alloxan administration may reflect in part renal insufficiency rather than the diabetic state per se.

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Acute hepatotoxic effects of alloxan might also modify the early metabolic effects on insulin insufficiency in such a manner as to either ameliorate or augment ketonemia during the initial period after alloxanization. Animals which survive through the fifth day after alloxan injection usually exhibit diabetes of varying severity characterized by partial or complete growth arrest, atrophy of the skin and coat, emaciation, hypertrophy of most of the viscera, and marked polyphagia, polyuria and glucosuria, but without significant ketonuria or ketonemia. Since these animals survive for long periods without ketosis, it has been assumed that the diabetes is relatively more mild than in other species or following pancreatectomy. In this report are presented the results of several recent experiments in our laboratory which suggest that this assumption may not be entirely justified. After treatment of non-ketotic alloxan diabetic rats for several weeks with protamine-zinc insulin, severe ketoacidosis developed upon withdrawal of insulin.

MATERIALS AND METHODS

Female Sprague-Dawley rats weighing 100–150 gm. from our colony were used in all experiments.[3] The diet given *ad libitum* consisted of standard Purina laboratory chow supplemented with vegetable leaves. Diabetes was produced by injection of alloxan monohydrate (35 mg./kg.) intravenously under ether anesthesia after a 48-hour fast. For some experiments severely diabetic animals were selected on the basis of growth rate and urine glucose excretion after a wait of 3 weeks following alloxanization to permit recovery from acute hepatic and renal toxicity. Protamine-zinc insulin (PZI, Lilly) was injected daily either in the morning or afternoon in doses as stated in the text.

pH determinations were carried out on freshly drawn arterial blood from the heart with a Cambridge glass electrode pH meter at 37° C; a standard solution of pH 6.97 was used as a reference.

Blood or urine glucose was determined by a glucose oxidase technique developed by Halsey (9) after deproteinization of the blood with $Ba(OH)_2$ and $ZnSO_4$ (10). Total ketone content of blood was determined after deproteinization using the method of Bessman and Anderson (11). Sodium B hydroxybutyrate was used as a standard, and the results expressed in mg. acetone/100 ml. blood. Qualitative tests for glucosuria were carried out with Clinitest tablets (Ames Company, Inc.) and tests for acetonuria with Acetest tablets (Ames Company, Inc.).

RESULTS

In the first experiment, diabetic rats were started on daily subcutaneous injections of 6 units of PZI approximately 48 hours after alloxanization. No attempt was made to assess the severity of the diabetes at the outset, beyond selection of animals which exhibited a 4+ urine test for glucose. The results are summarized in Table 1. After 41 days of treatment the non-injected control animals had gained only 41 gm. of body weight despite a high food intake, whereas the insulin-treated group had gained 144 gm. At

[3] Subsequent experiments have been done with male animals with essentially similar results.

the end of the period of therapy, the 6 unit daily insulin dose was equivalent to approximately 1.5 units per 100 gm. body weight. With this therapy, 24-hour glucosuria was reduced to very low levels, and the animals appeared healthy and exhibited none of the stigmata of alloxan diabetes. No symptoms of hypoglycemia were observed. When insulin was discontinued, a rapid loss of 30 gm. of body weight ensued within 48 hours. During this time there was only a slight increase in urine volume and 24-hour glucosuria, the amount remaining very small in comparison to the glucosuria of the untreated diabetic control animals (Table 1). Approximately 48 hours after the last injection of insulin positive urine tests for acetone

TABLE 1. RESPONSE IN BODY WEIGHT, GLUCOSURIA, BLOOD GLUCOSE AND BLOOD KETONES OF ALLOXAN DIABETIC RATS TO COMPLETE INSULIN WITHDRAWAL AFTER A PROLONGED PERIOD OF DAILY INJECTIONS

	No. of animals	Dose PZI, u./day	Duration, days	Body weight		Glucosuria,* gm./24 hr.
				Initial, gm.	Final, gm.	
Diabetic Control	7	0	—	97 ± 2.1†	138 ± 11.2	7.7 ± 0.6
Insulin Treated Diabetic	8	6	41	89 ± 3.4	233 ± 3.4	0.06 ± 0.037

	No. of animals	Hours after insulin withdrawal						
		24–48 hr.		48–72 hr.		86 hours		
		Body weight, gm.	Glucosuria, gm./24 hr.	Body weight, gm.	Glucosuria, gm./24 hr.	Body weight, gm.	Blood glucose, mg./100 ml.	Blood ketones, mg./100 ml.
Diabetic Control	7	137 ± 15.6	7.7 ± 0.8	141 ± 12.8	7.8 ± 1.2	133 ± 9.6	453 ± 15.7	6.4 ± 0.9
Insulin Treated Diabetic	8	212 ± 3.4	0.47 ± 0.3	203 ± 3.6	0.62 ± 0.4	202 ± 2.7	414 ± 57.4	101.4 ± 22.1

* Measured at the end of period of insulin injection.
† Standard error of the mean.

appeared in some of the animals, and after 72 hours most of them exhibited strongly positive tests for acetone. One of the animals died in coma, and others appeared to be moribund, with labored respiration and very low body temperatures. After 86 hours, all the animals appeared moribund and were sacrificed. Examination of these animals revealed marked gastric dilatation and an unusual pale yellow color of the kidneys and liver. Histological examination of kidney and liver specimens from several of these animals revealed extensive fatty infiltration of the hepatic and renal tubular cells similar to that described by Scow, Wagner, and Cardeza (2) in severe ketoacidosis. The blood glucose level in the treated group did not differ significantly from that of the diabetic control animals, but there was a striking elevation of the blood ketones to 101 mg./100 ml. in the treated group 86 hours after insulin withdrawal, compared with 6.4 mg./100 ml. in the non-injected controls.

Three of the diabetic control animals from the previous experiment were then started on daily insulin injections. After 22 days, therapy was halted

and these animals, which had previously exhibited no ketonuria and only a slight elevation of blood ketones, now developed typical signs of diabetic ketoacidosis. The blood ketone levels and arterial blood pH determinations revealed a marked ketoacidosis with pH values well below 7.0 (Table 2).[4]

From the preceding experiments, it is clear that ketoacidosis can develop in alloxan diabetic animals after a period of insulin treatment regardless of the interval after alloxan before treatment is begun. In another experiment insulin was withdrawn slowly after a period of treatment to determine whether the non-ketotic diabetic state could be restored. The animals were prepared as before, and the treated group received 6 units of PZI daily for a period of 42 days. The treated animals were then divided into 2 groups of 5 animals each. Insulin was stopped completely in the first group but was slowly withdrawn from the others, the dosage being decreased to 2 units

TABLE 2. EFFECT OF COMPLETE INSULIN WITHDRAWAL AFTER 3 WEEKS OF DAILY INJECTIONS UPON BLOOD KETONES AND ARTERIAL BLOOD PH OF NON-KETOTIC ALLOXAN DIABETIC RATS

No. of animals	Dose, PZI/day	Duration, days	Total weight gain, gm.	Glucosuria,* gm./24 hr.	96 hours after insulin withdrawal		
					Loss of body weight, gm.	Blood ketones, mg./100 ml.	Arterial blood, pH‡
3	6	22	103	0.11	48	144 ±7.7†	6.72 ±0.12

* Measured at the end of the period of insulin injection.
† Standard error of the mean.
‡ Average of 5 duplicate determinations on normal control animals anesthetized with pentobarbital sodium was 7.40 ±0.01.

PZI for 1 day, then 1 unit for 3 days, followed by 0.5 or 1.0 unit as needed to prevent overwhelming ketoacidosis. The group from which insulin was completely withdrawn developed severe ketonemia and acidosis within 96 hours, and all of those animals died or were sacrificed in a moribund state. The slow-withdrawal group continued to exhibit positive urine tests for acetone for approximately 10 days, the amount gradually decreasing. Two animals died during the withdrawal period. The remaining 3 gradually developed a large daily urine volume (70–125 ml.), and after 14 days no longer exhibited ketonuria as determined by Acetest tablets. These animals lost approximately 21 gm. of body weight during the withdrawal period. Most of this occurred during the first 2 days of withdrawal and was similar but less marked than in the complete-withdrawal group, which lost 29 gm. in 2 days. These animals were then started again on 6 units of PZI daily.

[4] In a subsequent experiment determinations of pH were carried out in three comatose animals which were moribund, responding only to painful stimuli. The pH values were 6.81, 6.81, and 6.80, corresponding to blood ketone levels of 84, 53, and 131 mg./100 ml. respectively. Another animal which was still alert but perceptibly less active than normal had a pH of 7.21 and blood ketone level of 123 mg./100 ml.

After 9 days of injections insulin was again withdrawn completely and ketonuria reappeared. Two of the three animals died with ketoacidosis within three days.

Although the dosage of insulin selected for the preceding experiments was well tolerated, very rapid weight gain was noted, especially during the first 2 weeks of treatment. Previous reports of dosage of PZI in alloxan

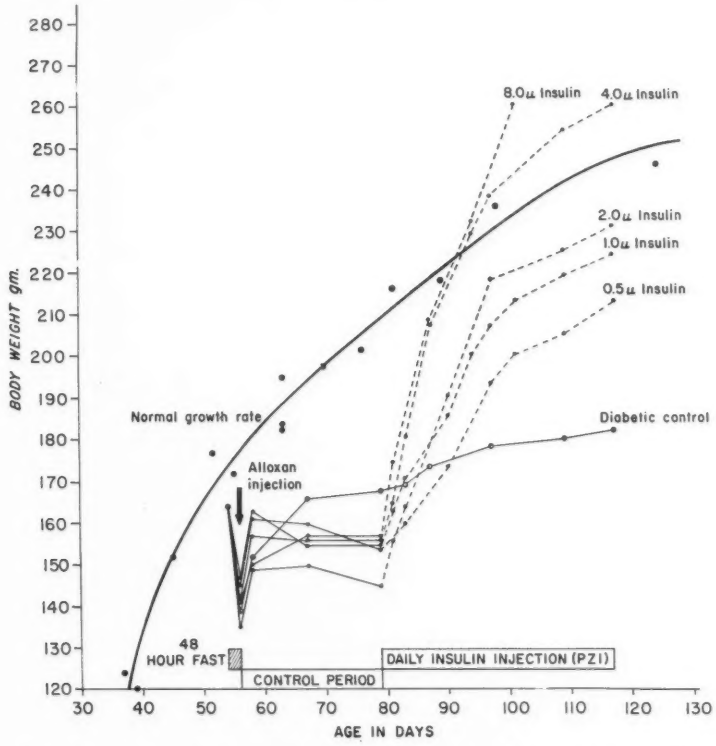

FIG. 1. Growth response of alloxan diabetic rats to various doses of PZI insulin. A curve of normal growth of non-diabetic animals is included for comparison.

diabetic rats indicated that fair control could be achieved with lower doses of approximately 1 unit per 100 gm. body weight (12, 13). An experiment was carried out to determine as nearly as possible a suitable dosage of insulin and to ascertain whether the development of ketoacidosis might be related to insulin antagonism arising from insulin overdosage.

Young rats were alloxanized as before. After a 22-day period, only animals with severe diabetes as judged by growth and glucosuria were selected for the experiment. Six groups of 6 animals each were treated with PZI at 5 dosage levels. Figure 1 depicts the doses employed and the results of

therapy. The diabetic control animals gained weight only very slowly. With 0.5 unit of insulin a significant effect on growth was noted, but these animals continued to excrete considerable quantities of glucose and were not restored to normal in terms of coat quality and body proportions. The animals treated with 1, 2, and 4 units daily appeared to be healthy, and growth was proportional to the dose of insulin up to 4.0 units daily. With 8.0 units daily, the growth rate was not faster than with 4.0 units, and, in this group, 2 animals died within the first 4 days and none survived 38 days. After 38 days of therapy, glucose excretion was determined. The diabetic control animals excreted an average of 11.5 gm. daily.

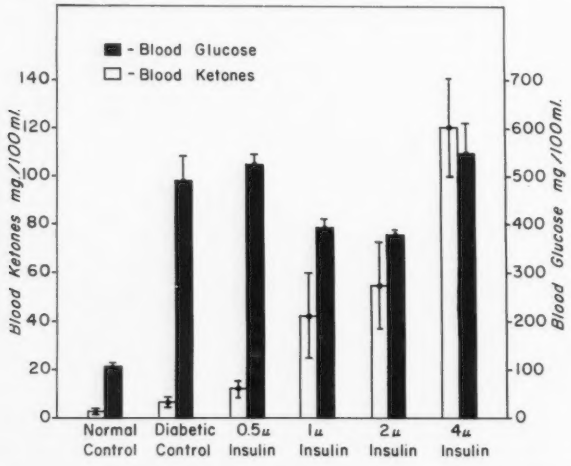

FIG. 2. Blood glucose and ketones 60 hours after complete withdrawal from insulin after daily injections for 38 days of PZI insulin in dosages as indicated.

The treated groups excreted average amounts of 8.7, 4.7, 1.8, and 0.9 gm., corresponding to insulin doses of 0.5, 1, 2, and 4 units daily. All the treated groups gained weight more rapidly during 38 days of therapy than did the nondiabetic controls during the same period (Fig. 1). However, only those animals receiving 2 units or more of insulin daily were restored to approximately normal body weight corresponding to their age. Restoration of normal body weight may be a better criterion of adequacy of therapy than rapidity of growth, since diabetic control was inadequate with lower doses of insulin (0.5 unit) which nonetheless induced rapid growth.

Insulin was withdrawn after injection for 40 days, and 60 hours later blood samples were taken for determination of glucose and ketones. Figure 2 illustrates the results. Although there was no significant difference in blood glucose levels in all the diabetic groups, marked ketonemia occurred only in animals that had had insulin treatment withdrawn and was propor-

tional in severity to the dosage of insulin. Even those animals treated with only 0.5 unit of insulin exhibited a statistically highly significant elevation of blood ketones as compared to the untreated diabetic controls. Thus the degree of ketonemia correlated well with the dosage of insulin as well as the body weight gain of the animals. The blood ketone level of the diabetic control animals was only slightly higher than that of normal control animals.

DISCUSSION

Numerous nutritional, biochemical, and endocrine factors play a role in the development of ketosis and ketoacidosis (14). It is generally accepted, however, that rather marked insulin insufficiency is necessary for the development of uncontrolled diabetic ketoacidosis. In our experiments, the most consistent results were obtained with severely diabetic animals selected several weeks after alloxanization on the basis of growth failure and marked glucosuria. Normal rats do not develop ketosis easily, and special diets high in fat or ketogenic substances are needed to produce significant fasting ketosis (15). The diet used in these experiments, however, was a standard laboratory diet high in carbohydrate and low in fat content (approximately 5%).

Elevated blood ketones have been observed in rats with long-standing alloxan diabetes (16), but the levels are usually only slightly higher than the levels in normal fasting rats. Serum lipids are also increased (17). Many of the early investigations of alloxan diabetes in rats mention the development of acetonuria and coma 3 or 4 days after administration of alloxan. Since azotemia and other acute metabolic derangements coexist with the diabetic state during the first 2 weeks after alloxan, these observations cannot be interpreted with certainty. In most instances, measurements of blood levels were not made, and those reported by Lackey *et al.* (18) indicated only a relatively mild ketonemia (29 mg./100 ml.).

Brodsky *et al.* (19), and Knowles and Guest (20) have described severe ketoacidosis in Rockland rats on withholding of food 24 hours after alloxan administration. The intravenous dose of alloxan employed by these investigators was somewhat higher than the usual dose and no observations of renal toxicity were made. In some experiments rats were treated with PZI or NPH insulin for one to two weeks after alloxan injection. Severe ketoacidosis developed when insulin was discontinued. Marked weight loss and symptoms similar to those observed in this study were noted. A fatal outcome was attributed to failure of the ketotic rats to eat, and in those instances where the animals survived insulin withdrawal, marked polyphagia and polyuria were noted. Such animals continued to lose weight slowly but did not exhibit ketosis. It is not clear from their results, however, whether the failure to eat was actually the cause or only a symptom

of diabetic ketoacidosis since no attempts were made to force feed the severely ketotic animals.

Beatty (5) made the interesting observation that during the first week after alloxanization, urinary excretion of ketone bodies is significantly elevated and later plateaus at a lower level. One plausible explanation for this initial ketonemia and ketonuria after alloxan might be that the sudden lack of insulin permits rapid mobilization and catabolism of body stores of fat. Subsequently ketosis abates when endogenous lipid sources have been exhausted. It would follow that the major role of insulin in reversibly restoring a ketotic diabetic state in the alloxanized rat might be through restoration of adequate reserves of fat in the depots, thus setting the stage for ketoacidosis when insulin is later withheld. No estimates of body fat were made in our experiments. However, simple inspection of the carcass of the ketotic rats revealed the presence of abundant adipose tissue whereas little or none was visible in the untreated, severely diabetic rats. Scow and coworkers (2) have suggested that the development of diabetic coma after removal of 99.5% of the pancreas is dependent on the body content of fat. In their experiments pancreatectomized animals with depleted fat stores did not develop ketoacidosis on prolonged fasting and survived longer than rats with increased fat stores. The latter died within 2 days in coma.

Janes and Friedgood (21) demonstrated that adrenalectomy alleviated ketosis and decreased the severity of diabetes in rats several days after alloxan injection. More recently, Scow, Chernick, and Guarco (22) have demonstrated that cortisone alone permits the development of severe ketoacidosis in hypophysectomized, adrenalectomized, and depancreatized rats. Field (23) has observed that insulin therapy prevents adrenal hypertrophy in the alloxan diabetic rat. Hypoglycemia from excess insulin on the other hand may produce increased adrenocortical secretion in normal rats (24), and this possibility must be considered as a contributing factor to ketoacidosis in the insulin-treated diabetic rats. However, significant ketonemia appeared even in animals which had received doses of insulin too small to completely reverse the diabetic picture.

Apparently permanent diabetes induced by alloxan must involve destruction of at least 95% of the functioning β cells mass (4, 7). Measurements of pancreatic insulin content by Wrenshall, Collins-Williams, and Hartroft (25) indicate amounts less than 10% of normal 48 hours after alloxan and, in some instances, amounts as low as 2.3% of normal. Thus in severe alloxan diabetes the degree of insulin deficiency may not differ greatly from that after "total" pancreatectomy. The absence of ketosis does not necessarily indicate a less severe diabetic state but perhaps reflects more significantly the availability of endogenous lipids for fatty acid oxidation.

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SEX DIFFERENCES IN ADRENAL CORTICAL SECRETION IN THE RAT[1]

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ABSTRACT

Ether anesthesia administered as a standardized stress produced higher and more persistently elevated plasma corticosterone concentrations in female rats than in male animals. Similar effects were obtained following administration of ACTH. The biological half-life of corticosterone in female rats was found to be 13 minutes compared to 20 minutes in male animals. Female rat liver homogenates metabolized the A ring of corticosterone and that of cortisone more rapidly *in vitro* than did male tissue. No sex difference was observed in the rate of metabolism of the sidechain of corticosterone although male liver homogenates reduced the sidechain of cortisone more rapidly than did female tissue. The concentration of corticosterone in the adrenal vein of the female rat is 2.5 times that in the male. These findings are interpreted to signify increased sensitivity of the female adrenal gland to stimulation by ACTH. The higher plasma corticosterone levels observed after ACTH or stress in female rats are not explained by the sex differences in the rate of clearance and metabolism of the steroid. An additional possibility to be considered is that the female pituitary gland is more responsive to stress with respect to ACTH release.

SEX differences have been described in the rate of metabolism of corticosterone and cortisone by rat liver tissue *in vitro* (1, 2). The present study was undertaken to explore the possibility of additional differences in adrenal cortical secretion between male and female rats *in vivo*.

METHODS

Adult male and female albino rats of the Sherman strain were used in all experiments. The animals were maintained in a constant temperature room on a diet consisting of Purina Laboratory Chow and water *ad lib*. Plasma corticosterone concentrations were measured in 0.1–0.5 ml. samples by the method described by Guillemin *et al.* (3). Rats were decapitated with a guillotine (Harvard Apparatus Company) within 20 seconds after handling was begun and blood from the trunk collected in a 50 ml. beaker containing 0.1 ml. of heparin.

Effects of ether anesthesia. A standardized ether stress was administered according to the method of Wells *et al.* (4). Unstressed rats were decapitated at the beginning of the experiment and stressed animals decapitated 5, 15, 30, 60, 90 and 120 minutes post-anesthesia.

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Effects of ACTH administration. An aqueous solution of ACTH, 50 milliunits/0.1 ml., was administered intraperitoneally at a dose of 50 milliunits per 100 gm. body weight. Uninjected rats were decapitated at once and injected animals at intervals of 15, 30, 60 and 120 minutes. Additional animals were injected with saline and decapitated 30 minutes later.

Effects of corticosterone administration. All the animals were adrenalectomized 16 hours prior to use. Corticosterone, 2 mg./ml. 20% ethanol in water, was injected via the femoral vein under ether anesthesia at a dose of 500 μg per 100 gm. body weight. Animals were decapitated 5, 15, 30, 60, 90 and 120 minutes following injection.

Hepatic metabolism of steroids in vitro. Rats were decapitated and exsanguinated. A portion of liver was removed and homogenized in 0.25 M sucrose made 0.04 M with nicotinamide. Sidechain and ring A reduction of 150 μg of added cortisone were studied according to the method of Troop (1) without modification. In addition, similar experiments were made with the addition of 150 μg of corticosterone. The glyoxal reaction described by Lewbart and Mattox (5) was applied prior to the Porter-Silber reaction to measure sidechain reduction.

Adrenal vein corticosterone concentrations. Adrenal vein blood was obtained 5–15 minutes after induction of Nembutal anesthesia. Following laparotomy, a #27 gauge needle was passed through the left renal vein into the adrenal vein and threaded carefully above the level of any extra-adrenal branches. Blood was withdrawn slowly over a 5-minute period into a 1 ml. tuberculin syringe rinsed with heparin. Male and female rats of varying weights were selected according to previous experience so as to obtain approximately equal mean adrenal weights.

RESULTS

Unstressed male rats demonstrated mean plasma corticosterone concentrations of 8.0 ± 2.0 μg/100 ml. and 10.1 ± 0.7 μg/100 ml. on two occasions (Figs. 1, 2). In corresponding experiments, plasma corticosterone concentrations in unstressed female rats were 15.7 ± 0.9 μg/100 ml. and 20.8 ± 6.6 μg/100 ml. respectively. These values suggest that female rats have a higher resting plasma steroid level than do male rats. However, the possibility remains that a rise in plasma corticosterone concentration occurred in the female animals during the 20-second period of handling prior to decapitation.

Response to ether anesthesia. Following the application of a standardized ether stress, male rats showed a prompt rise in plasma corticosterone concentration to a peak of 53.8 ± 4.7 μg/100 ml. at 30 minutes (Fig. 1). Plasma levels fell thereafter and approximated resting level at 120 minutes post-stress. Female rats also demonstrated a peak plasma steroid level 30 minutes after the application of the ether stress. However, the plasma concentration attained was strikingly higher, 93.3 ± 14.5 μg/100 ml., and re-

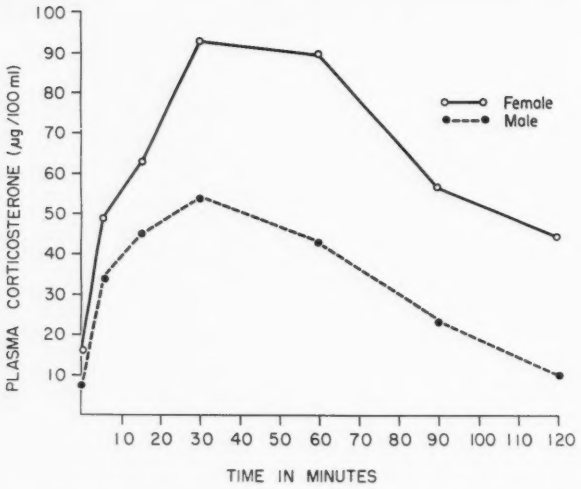

FIG. 1. Plasma corticosterone concentrations following a standardized ether stress in male and female rats. Each point represents the mean of 5 observations.

mained essentially unchanged at the end of 60 minutes. The value, 43.7 ± 3.9 μg/100 ml., observed at 120 minutes was significantly higher than the resting level.

Several hypotheses are suggested by these data. The higher plasma corticosterone levels observed after ether stress in female rats may be explained by: a) a sex difference in the responsiveness of the adrenal gland to the

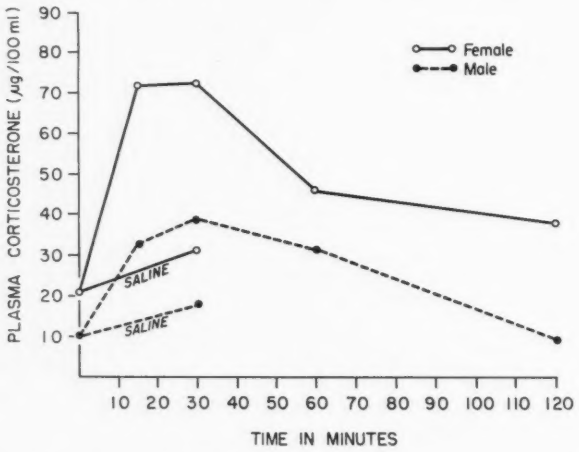

FIG. 2. Plasma corticosterone concentrations following intraperitoneal injection of 50 milliunits of ACTH per 100 gm. body weight in male and female rats. Each point represents the mean of 5 observations.

same amount of ACTH stimulation; b) a difference in the rate of clearance and metabolism of plasma corticosterone in male and female rats; and, c) a sex difference in the amount of ACTH released in response to stress. The next experiment was performed to explore the first of these hypotheses further.

Response to exogenous ACTH. Administration of ACTH to male rats resulted in a peak plasma corticosterone level of 39.2 ± 4.3 μg/100 ml. 30 minutes after injection with return to the resting value at 120 minutes (Fig. 2). Female rats reached a level of 72.2 ± 3.0 μg/100 ml. at 15 minutes with essentially no change at 30 minutes. The plasma steroid concentration remained elevated at 120 minutes. The sex difference in response to ACTH is comparable to the difference observed following ether anesthesia (Fig. 1). Since intact animals were employed, the possibility exists that the injection of ACTH was stressful of itself and that release of endogenous ACTH was stimulated. This was evaluated by the administration of saline in similar fashion to the injection of ACTH. Thirty minutes following such injection, male rats showed a plasma corticosterone level of 18.2 ± 3.3 μg/100 ml., whereas the corresponding value in female rats was 31.2 ± 9.3 μg/100 ml. These findings suggest that the stress of injection *per se* did not contribute significantly to the sex difference observed after ACTH administration.

The data support the hypothesis that the adrenal gland of the female rat is more sensitive to ACTH than is the male adrenal gland. However, an alternative explanation, equally applicable to the effects obtained after stress or after ACTH is that a slower rate of plasma clearance and metabolism of corticosterone occurs in female rats. This hypothesis was evaluated in the following experiments.

Corticosterone half-life. The clearance of injected corticosterone was studied next and the results shown in Figure 3. The biological half-life in male rats was 20 minutes whereas the comparable value in female animals was 13 minutes. These times differ significantly at the 1 percent level of confidence. The rats employed in this experiment were injected 16 hours after adrenalectomy to eliminate variation due to endogenous steroid secretion. Uninjected adrenalectomized rats of both sexes revealed similar residual fluorescence values in the plasma corresponding to steroid concentrations of 5–6 μg/100 ml., levels comparable to those already reported (6). The half-life values obtained in this experiment agree substantially with those described recently by Herbst *et al.* (7) with the use of tracer amounts of corticosterone-4-C^{14}.

Hepatic metabolism of steroids. Corticosterone and cortisone were added to liver homogenates according to the *in vitro* technique of Troop (1), and the results are summarized in Table 1. Liver tissue from female rats reduced the A ring of either steroid more rapidly than did male liver tissue in conformance with the findings of previous studies (1, 2, 7, 8). Conversely,

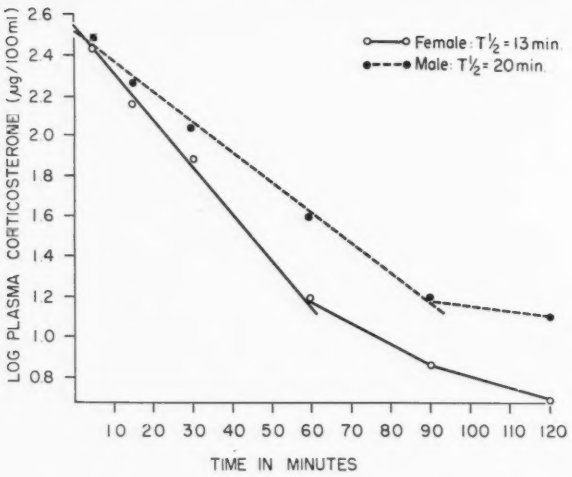

FIG. 3. Logarithm of plasma corticosterone concentrations following intravenous injection of 500 μg corticosterone per 100 gm. body weight in male and female rats. Each point represents the mean of 5 observations.

the C-20 ketone of cortisone was metabolized more effectively by male liver tissue confirming the observations of Troop (1, 8). On the other hand, no sex difference was observed in the rate of reduction of the sidechain of corticosterone.

Adrenal vein secretion. Finally, the concentration of corticosterone was measured in the adrenal vein of male and female rats. Differences in the rate of plasma clearance or of hepatic metabolism of steroid may be excluded as significant contributory factors under these circumstances. The mean level observed in female animals was 2.5 times higher than that found in male rats (Table 2). This finding may not be correlated with a sex difference in adrenal size since adrenal weights were approximately equal. A moderate to severe degree of stress must be assumed as one of the condi-

TABLE 1. REDUCTION OF THE 20-KETONE AND Δ^4-3-KETONE GROUP OF CORTICOSTERONE AND CORTISONE IN MALE AND FEMALE RATS

Group	Micrograms reduced	
	Male	Female
Corticosterone Sidechain[1]	24.4 ± 4.3[4] (12)	21.9 ± 3.7 (12)
Corticosterone Ring A[2]	37.0 ± 5.1 (10)	82.2 ± 2.5 (13)
Cortisone Sidechain[3]	76.5 ± 8.7 (14)	14.7 ± 3.9 (6)
Cortisone Ring A[2]	42.5 ± 7.9 (13)	80.3 ± 3.3 (7)

No. of animals studied indicated in parentheses.
[1] Porter-Silber reaction on glyoxal derivative.
[2] Ultra-violet absorption at 240 mμ.
[3] Porter-Silber reaction.
[4] Mean ± standard error.

TABLE 2. PLASMA CORTICOSTERONE CONCENTRATIONS IN THE ADRENAL VEINS OF MALE AND FEMALE RATS

Sex	No. of rats	Body wt. (gm.)	Left adrenal wt. (mg.)	Corticosterone (μg/100 ml.)
Male	12	258 ± 8[1]	19.5 ± 1.5	422 ± 46
Female	17	165 ± 4	22.3 ± 1.0	1014 ± 100

[1] Mean ± standard error.

tions of this experiment in view of the anesthesia, the laparotomy and the manipulation of the abdominal viscera necessary to permit cannulation of the adrenal vein.

DISCUSSION

Female rats demonstrated markedly higher and more persistently increased plasma levels of corticosterone after stress than did male animals. Similar results were obtained following administration of ACTH suggesting that the greater secretion of corticosterone observed in female rats is due to greater adrenal responsiveness to tropic stimulation. Certainly plasma clearance and hepatic metabolism of corticosterone may be excluded as significant contributory factors. In fact, the shorter biological half-life and more rapid hepatic reduction of corticosterone characteristic of the female rat might reasonably have been expected to result in more rapid restoration of resting plasma steroid levels rather than the prolonged elevations actually found. Although hepatic regulation of adrenal cortical secretion has been shown to be an important consideration in the evaluation of a number of experimental treatments (7), such regulation is not applicable to the present findings. Both stress and ACTH stimulation were administered acutely and significant changes in hepatic enzyme activity could hardly have occurred within the brief time span involved. In view of the more rapid rate of steroid clearance from plasma in the female rat, it is apparent that the almost two-fold difference in peak plasma corticosterone concentration observed after stress or ACTH can only be accounted for by a markedly greater secretion of the steroid. This is supported by the finding of a higher concentration of corticosterone in the adrenal vein of the female animals despite equivalent adrenal secretory mass.

An additional explanation of the sex difference in adrenal steroid secretion after ether anesthesia is that female rats release a greater quantity of ACTH in response to stress than male rats. In other words, the increased responsiveness of the female adrenal cortex may be associated with increased responsiveness of the female pituitary gland with respect to ACTH secretion. The latter hypothesis was not tested in the present study. However, Gemzell has reported increased secretion of ACTH in male rats following administration of estradiol (9). Gompertz suggested that estradiol affects both the hypophysial secretion of ACTH and adrenal cortical sensi-

tivity to ACTH based upon adrenal weight changes in intact and castrated rats (10).

Sex differences in the metabolism of cortisone by rat liver homogenates have been reported by Hagen and Troop (1, 8). Female rats reduced the A ring more rapidly than did male animals whereas male rats reduced the C-20 ketone more rapidly. A variety of experimental treatments to either sex was shown to modify the rates of reaction at both sites on the steroid molecule. These authors concluded that the sex difference in sidechain metabolism demonstrated by rat liver homogenates *in vitro* may have functional importance *in vivo* comparable to the well-established significance of ring A metabolism. It is of interest, therefore, that in the present study no difference between male and female rats was observed in their metabolism of the sidechain when corticosterone was employed as the substrate. The physiologic significance of the sex difference in sidechain reduction observed with cortisone is subject to question in view of the fact that corticosterone is the predominant steroid hormone secreted by the rat adrenal gland. Hitherto, studies involving reduction of the C-20 ketone have been confined to compounds with a dihydroxy-ketone configuration on the sidechain due to the limitations of the Porter-Silber reaction. Preparation of the glyoxal intermediate as described by Lewbart and Mattox (5), extends the range of application of the Porter-Silber reaction to include 17-desoxy compounds such as corticosterone.

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SULFURYLATION OF ESTROGENS BY THE HUMAN FETUS[1]

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ABSTRACT

The metabolism of estrone-16-C^{14} was studied in 3 human fetuses of 3 to 5 months gestation. About one-third was converted to water-soluble metabolites. By enzymatic hydrolysis and paper chromatography these were shown to be virtually all estrogen sulfates. A small amount of the estrone was converted to estradiol-17β but no estriol was detected. Estrone-16-C^{14} was sulfurylated efficiently in the 105,000$\times$g supernatant fraction of guinea pig placenta, but not in term human placenta.

THE human fetus converts estrogens to water-soluble metabolites at an early stage in gestation (2), although the fetus is deficient in enzymes necessary for glucosiduronidation (3). Our interest in the identity of these metabolites stems from the observation that in the guinea pig the placenta poses an effective barrier to estrogen sulfates and glucosiduronates under conditions in which the unconjugated estrogens pass freely (4).

In the present study the metabolism of estrone-16-C^{14} was investigated in 3 human fetuses of 3 to 5 months gestation. In each instance virtually all of the recovered water-soluble metabolites were sulfates. In contrast to a previous investigation in the guinea pig (4) which demonstrated sulfurylation by the placenta, no significant sulfurylation could be demonstrated by perfusion of a human placenta of 3 months gestation.

MATERIALS AND METHODS

The human fetuses were available to us immediately after delivery by hysterotomy. Abortions were performed for medical indications in the mother. The fetuses appeared normal. The estimated periods of gestation were from 3 to 5 months. Estrone-16-C^{14} was dissolved in a few drops of ethanol and diluted to 1 ml. with 0.9% sodium chloride. The solution was injected into the umbilical vein of the fetus. After 45 minutes at 37° C, during

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[2] Markle Scholar in Medical Sciences.

which time the heart continued to beat but no breathing was discernible, the viscera or the total fetus was extracted with 80% ethanol in a blender.

The placenta from the 3 months pregnancy was perfused with 30 μg of estrone-16-C^{14} in cord blood plasma by injecting the material by syringe into an umbilical artery. The plasma that flowed out of the umbilical vein was recovered and injected a second time. The placenta and plasma were extracted individually with 80% ethanol.

Sulfurylation of estrone-16-C^{14} was studied using the supernatant of guinea pig and human placental homogenates prepared in KCl solution by centrifuging at 105,000×g for 45 minutes. Incubation was for 2 hours at 37° C in the presence of adenosine triphosphate (ATP) and inorganic sulfate (5). Following incubation, 4 volumes of absolute alcohol were added and the mixture was filtered.

Analyses.—In the three month pregnancy, the entire fetus was extracted and analyzed. In the four month pregnancy, the head, most of the blood and a small fraction of several vital organs were removed for pathological study, the rest of the fetus being available for analysis. The blood and viscera of the five month fetus were analyzed.

The alcoholic extracts were evaporated *in vacuo* at room temperature. The residue was partitioned between ether and water to give the unconjugated and conjugated fractions respectively. Preliminary identification of conjugates was made by treating aliquots with the hydrolytic enzymes β-glucuronidase[3] and phenolsulfatase[4] by methods described previously (6). The residual conjugate fractions were identified further by paper chromatography and enzymatic hydrolysis of the eluted radioactivity. First the conjugates were partitioned between butanol and 0.1N sodium hydroxide. The butanol fraction which contained the sulfates was distilled under vacuum and the residue was chromatographed on paper using the solvent system, butanol-toluene (10:10)/ammonia-water (2:18) (7). A parallel standard contained estrone-16-C^{14} sulfate. The single radioactive zone[5] was eluted with methanol. The methanol was distilled in vacuum. An aqueous solution of the residue was hydrolyzed with phenolsulfatase. The radioactivity extracted with ether is presumed to originate from sulfates.

Ethereal solutions of the unconjugated estrogens and those obtained after enzyme hydrolysis were treated in the same manner. The ether was removed in a stream of nitrogen. The non-polar metabolites (estrone and estradiol-17β) were separated from the polar metabolites (estriol and 16-epiestriol) by silica gel chromatography (8). There was no radioactivity in the latter. The estrone-estradiol-17β fraction was chromatographed on paper using the solvent system benzene-formamide (9). After 4 hours, zones of radioactivity corresponding to estrone and estradiol-17β were eluted. After dilution with 20 mg. of carriers each was crystallized from 95% ethanol to constant specific activity. The benzoates were prepared and each was crystallized from ethanol to constant specific activity. The percentages of estrone and estradiol-17β were calculated.

The 4-month fetal extracts were analyzed differently. Estrone and estradiol-17β carriers were added directly to the unconjugated fraction and the ether extract of the phenolsulfatase hydrolysate. After chromatography on silica gel the estrone-estradiol-17β fraction was separated by the Girard reaction (10). The estrogen was crystallized to constant specific activity and converted to the benzoate which also was crystallized to constant specific activity.

Radioactivities were determined in either the D 46A Nuclear windowless flow gas counter (50% efficiency) or the D 47 Nuclear micromil window counter (25% efficiency). Counts were corrected to infinite thinness.

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[3] Ketodase, Warner-Chilcott Laboratories, New Jersey.

[4] Mylase P., Wallerstein Laboratories, New York.

[5] Determined in a windowless chromatogram scanner purchased from Atomic Accessories, New York.

TABLE 1. CONVERSION OF ESTRONE-16-C^{14} TO WATER-SOLUBLE METABOLITES BY HUMAN FETUSES*

Estimated gestation months	Fetal weight, gm.	c.p.m. extracted	Percent water-soluble
3	70†	8.0×10^5	31
4	342‡	8.0×10^5	8
5	440[a]	1.3×10^6	35

* About 0.1 mg. of estrone-16-C^{14} was injected into the umbilical vein and 45 minutes later radioactivities in fetuses were analyzed.
† Entire fetus analyzed.
‡ Head, most of blood and parts of vital organs not available for analysis.
[a] Blood and viscera were analyzed.

RESULTS

The experiments were terminated 45 minutes after the injection of estrone-16-C^{14} into the umbilical vein of the human fetus. By this time about one-third of the recovered radioactivity had already been converted into a water-soluble form (Table 1). The water-soluble fractions were almost completely hydrolyzed by phenolsulfatase (Table 2). The hydrolysis was significantly inhibited by inorganic phosphate, supporting the identification of the conjugates as sulfates (11). The identification was confirmed further by paper chromatography of the water-soluble fraction which produced one radioactive zone corresponding in mobility to a known standard estrone-16-C^{14} sulfate. Glucosiduronates were barely detectable.

The ether-soluble fractions derived from hydrolysis with phenolsulfatase were analyzed by the reverse isotope dilution method. The unconjugated ether-soluble fraction was analyzed similarly. In each sample estrone comprised the major fraction (Table 3) and there was a small amount of estradiol-17β. No estriol was detected.

The placenta of approximately 3-months gestation was perfused for 2 cycles with 30 micrograms of estrone-16-C^{14}. About 2% of the radioactivity

TABLE 2. HYDROLYSIS OF FETAL WATER-SOLUBLE RADIOACTIVITY BY PHENOLSULFATASE AND β-GLUCURONIDASE

	Estimated gestation—months		
	3	4	5
Phenolsulfatase*	94%	90%	95%
Control	20	0	0
Phenolsulfatase and Phosphate	48	18	16
β-Glucuronidase†	4		2
Control	3		1

* Aliquot of water-soluble radioactivity was incubated with Mylase P., 0.5 mg. per ml. at 50° C for 18 hours at pH 6.2 (acetate buffer). The control experiment was carried out as above except that enzyme was omitted. To another aliquot was added inorganic phosphate to final concentration of 0.13 M resulting in a reduction of hydrolysis.
† Aliquot was incubated with Ketodase, 500 Fishman units per ml. at 37° C for 18 hours at pH 5.0. In the control experiment enzyme was omitted.

TABLE 3. FETAL METABOLITES OF ESTRONE-16-C^{14} AS DETERMINED BY REVERSE ISOTOPE DILUTION*

Numbers represent percent of total extracted radioactivity

	Estimated gestation—months		
	3	4	5
Sulfates			
Estrone	15	7	9
Estradiol-17β	2	1	1
Unconjugated			
Estrone	†	92	35
Estradiol-17β	†	0.2	2

* Estrone and estradiol-17β carriers were added directly to the phenolsulfatase hydrolysate and the unconjugated extract of the 4 month fetus. In the 3 and 5 month studies carriers were added after the metabolites were purified by column and paper chromatography. See text for details.

† Unconjugated metabolites were not determined.

in the perfusate and placenta was rendered water-soluble but no sulfates were detected by enzyme hydrolysis.

The 105,000×g supernatant from term placenta homogenate was incubated with the necessary sulfurylating co-factors. Only 1.5 to 2.5% of the radioactive estrone was rendered water-soluble. Most of this was hydrolyzed by phenolsulfatase. This contrasts to the efficient sulfurylation by guinea pig placental homogenates (Table 4).

DISCUSSION

The data presented demonstrates the ability of the human fetus to conjugate estrogens in the first and second trimesters. In two of the 3 experiments (Table 1) about one-third of the administered estrone was rendered water-soluble. In the third experiment the result of 8% cannot be evaluated because only part of the fetus was available for analysis. In all cases the conjugates were virtually all sulfates. Preliminary identification was made by hydrolysis with Mylase P., an impure enzyme preparation. Confirmation was obtained by (a) the inhibition of hydrolysis by phosphate

TABLE 4. SULFURYLATION OF ESTRONE-16-C^{14} BY HOMOGENATES OF HUMAN AND GUINEA PIG PLACENTAS

Placenta	Estimated gestation	μg incubated	Percent water-soluble	Percent of water-soluble as sulfates*
Human	term	6	1.4	53
Human	term	6	2.5	73
Guinea Pig	middle	37	14	92
Guinea Pig	$\frac{1}{3}$	6	98	84

24 ml. of homogenate from 5 gm. of placenta were incubated for 2 hours with 0.001 M sodium pnosphate buffer, pH 6.5, 0.12 M KCl, 0.015 M $MgSO_4$ and estrone-16-C^{14}. Homogenates are the supernatant fractions after centrifuging at 105,000×g. for 45 minutes.

* Determined by enzyme hydrolysis.

(11), (b) extraction of the conjugates from 0.1N sodium hydroxide with butanol, and (c) paper chromatography.

The failure to obtain significant amounts of glucosiduronates is not unexpected. Fetal tissue is deficient in uridine diphosphate glucosiduronic acid (UDPGA) as well as the enzyme which transfers glucosiduronic acid from UDPGA to steroids and other substrates (3).

Metabolism by the placenta has also been studied. The sulfurylation of estrogens by the perfused guinea pig placenta had been demonstrated previously (4). Perfusion of one human placenta of 3-month's gestation with estrone-16-C^{14} yielded no detectable sulfates. In contrast to results with early guinea pig placenta, homogenates of human term placenta sulfurylated estrogens poorly, if at all. The small water-soluble fraction was hydrolyzed by phenolsulfatase but there was insufficient radioactivity for confirmation by paper chromatography. Although comparisons of placentas of comparable gestations were not made in these studies, it appears that there is a distinct species difference in this respect.

Analysis of the conjugated and unconjugated estrogen fractions revealed small amounts of estradiol-17β in addition to the estrone. This is in agreement with earlier observations that slices of human fetal tissue interconvert these estrogens (12). Estriol was not detected. The origin of the large amounts of estriol found in pregnancy is controversial. There is evidence that fetal metabolism is responsible for at least part of its production. It is reported that the levels of estriol in fetal blood exceed that of human maternal blood five-fold (13). In addition the conversion of estradiol-17β to estriol by human fetal liver has been observed (14). Perhaps the failure to obtain estriol in the present experiments is a result of the anoxic conditions of the experiments.

The quantitative differences in accounting for the metabolites of the injected estrone shown in Table 3 may be the result of differences in methodology. In the 4-months fetus estrone and estradiol-17β carriers were added directly to the unconjugated fractions and the phenolsulfatase hydrolysates. In this experiment virtually all the radioactivity was accounted for, so that the major metabolites of estrone can be identified with certainty. In the other 2 experiments there were significant losses during the analytical procedures. Therefore, one can only state that the pattern of metabolites in the *recovered* radioactivity was the same.

The significance of sulfurylation by the fetus cannot be evaluated adequately until more information on the placental transfer of estrogens and their conjugates is available and until the broader question of the endocrinological consequences of conjugation is answered. Short term experiments in the guinea pig have demonstrated a relative impermeability of the placenta to sulfate conjugates suggesting that conjugation in the fetal side of the placenta could interfere with excretion of these compounds. It

is not known whether these results are applicable to the human or whether prolonged experiments might reveal limited transfer of the conjugates. The presence of an effective de-sulfurylating mechanism in the fetus or placenta could resolve the problem by converting the sulfates back to the unconjugated and permeable forms. Under these circumstances it is possible that sulfurylation merely prolongs the estrogenic effect. Arylsulfatase activity has been demonstrated in human embryonic tissue and placenta in *in vitro* studies (15).

ADDENDUM

Since the submission of this manuscript evidence has been presented by Dr. Egon Diczfalusy at the Laurentian Hormone Conference (Mont Tremblant, Canada, September, 1960) that the human fetus converts estrogens to sulfates and, to a lesser extent, to the glucosiduronate conjugates.

Acknowledgments

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THE RELATIONSHIP OF URACIL, OROTIC ACID AND THIOURACIL IN MAMMALIAN THYROID TISSUE[1]

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ABSTRACT

Neither uracil nor orotic acid are able to reverse the anti-thyroid effects of thiouracil in the rat or in sheep thyroid slices. Furthermore, these pyrimidines do not inhibit either the metabolism of thiouracil in the thyroid of the rat, or the transsulfuration of the sulfur of thiouracil to the protein of a sheep thyroid particulate preparation.

IT HAS been suggested that the anti-thyroid effect of 2-thiouracil may be due to its interference with nucleic acid metabolism. This proposal arose from observations made with plants (1), bacteria (2, 3) and viruses (4, 5) in which the inhibitory action of thiouracil was overcome by uracil, a pyrimidine base of ribonucleic acid (RNA). The concept gained further significance when a nucleoside of thiouracil was prepared enzymatically (6) and thiouracil was found to be incorporated into the RNA of tobacco mosaic virus (TMV) (7, 8, 9). It is the purpose of this study to investigate the effects both of uracil and of orotic acid on the inhibitory action and the metabolism of thiouracil in mammalian thyroid tissue.

METHODS

Materials:

2-Thiouracil-S^{35}, uracil-2-C^{14} and orotic-6-C^{14} acid were obtained from the New England Nuclear Corporation. The specific activity of the thiouracil was 30 millicuries per millimole; that of uracil and orotic acid was 3.1 millicuries per millimole. The chromatographically pure compounds were dissolved in dilute potassium hydroxide, final pH 8.0–8.5 and stored at −20°. Radioactive iodide (I^{131}), essentially carrier free, was obtained from the Atomic Energy Commission, Oak Ridge, Tennessee.

Animal Experiments:

(a) *With thiouracil-S^{35}.*—Male Sprague-Dawley rats weighing 100 to 125 gm. were maintained on finely ground Purina fox chow and water *ad libitum*. The radioactivity labeled compound was administered intraperitoneally as a single dose, 20 micromoles (μmoles). At intervals, the animals were killed by exsanguination. Aliquots of tissue

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homogenates, the serum, and thiouracil-S^{35} standards were assayed for total S^{35}-radioactivity (10).

The quantity and the nature of the S^{35}-metabolites were determined by paper chromatography and by paper electrophoresis (10, 11). Carrier thiouracil was identified by ultraviolet quenching. A typical chromatogram is shown in Figure 1.

(b) *With uracil-2-C^{14} and orotic-6-C^{14} acid.*—These compounds were also admin-

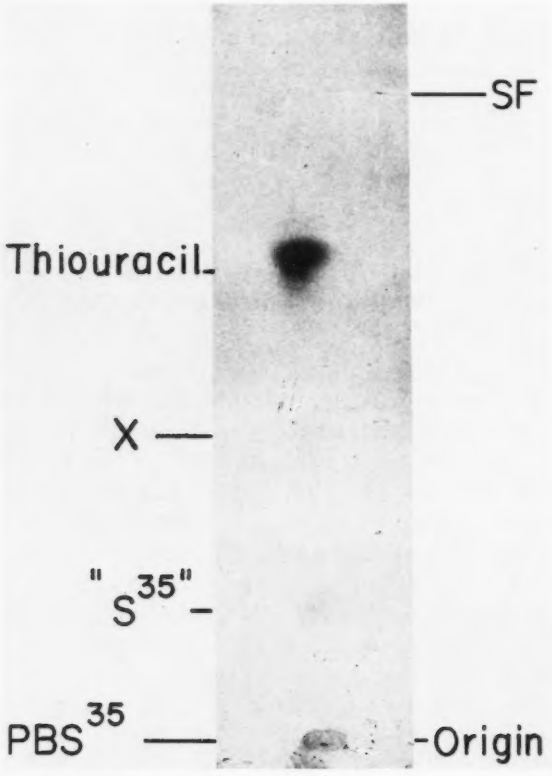

FIG. 1. Radioautogram of a paper chromatogram run in an ascending solvent system of ethanol-1.0 M ammonium acetate, pH 7.5 (7.5:3). This solvent separates a radioactive spot which remains at the origin (protein-bound S^{35}, PBS^{35}), an inorganic S^{35} spot with an Rf of 0.23 which is a mixture of thiosulfate and sulfate, thiouracil with an Rf of 0.75, and an unknown spot with an Rf of 0.50. Thiosulfate and sulfate are separated by paper electrophoresis (11).

istered intraperitoneally to the rat as a single dose, 20 μmoles. Unless otherwise stated, the protocol and analytical methods were as described for thiouracil-S^{35}.

The C^{14} metabolites were determined by paper chromatography. Carrier uracil and orotic acid were identified by ultraviolet quenching. Uracil has an Rf of 0.67 and orotic acid an Rf of 0.38 in the solvent system of ethanol-1 M ammonium acetate.

(c) *With non-radioactive thiouracil, uracil and orotic acid.*—Various concentrations

and combinations of thiouracil, uracil, and orotic acid were thoroughly mixed with Purina fox chow diet. The animals were weighed at the beginning and also several times during the course of each experiment which lasted two weeks. The amount of food consumed was measured and was found to be about equal for each group of animals. At the end of the two-week period, the animals were killed by exsanguination. The thyroids were removed, cleaned, and weighed on a Roller Smith torsion balance. The serum protein-bound iodine (PBI) was determined by a modification of Chaney's method (12). The ratio of the concentration of inorganic radioactive iodide in the thyroid to that in the serum, *i.e.*, the "T/S ratio" was measured according to the method of VanderLaan and VanderLaan (13).

Slice Experiments. Fresh sheep thyroid slices weighing about 200 mg. were incubated in standard 25 ml. Erlenmeyer flasks with Krebs Ringer Bicarbonate buffer, pH 7.4, 0.5 microcurie of radioactive iodide (I^{131}), and various concentrations of thiouracil and/or orotic acid. After incubation, the slices were then removed from the media, pressed gently against the side of the flask, and homogenized with 1.0 ml. of chilled 10% trichloracetic acid per 100 mg. of tissue. The uptake of I^{131} in the slices was determined by counting the entire homogenate in a well-type scintillation counter and comparing the radioactivity with an appropriate standard. Total radioactivity recovered was greater than 95%. The radioactivity in the homogenate was fractionated into inorganic iodide (I^{131}) and protein-bound iodine (PBI^{131}) by trichloracetic acid precipitation and washing.

Thyroid Cytoplasmic Particulate Experiments:

A sheep thyroid cytoplasmic particulate fraction was prepared by methods previously described (14). This corresponded to a pellet obtained from the 8,500×g supernatant centrifuged at 105,000×g for 60 minutes in the No. 40 rotor of the Spinco Preparative Ultracentrifuge. Pellets were suspended by homogenization in 0.1 M Tris, pH 7.4. One ml. of this suspension contained the particles from 1.0 gm. of thyroid tissue. It has been shown previously that this preparation carries out a transsulfuration of the sulfur of thiourea to thyroid protein (14).

All experiments were performed at least two or three times. Each table represents a typical experiment.

RESULTS

Uptake and Metabolism of Thiouracil-S^{35}

It is evident from Table 1 that thiouracil reaches the thyroid within minutes of its intraperitoneal administration to the animal, and that there

TABLE 1. THE CONCENTRATION OF THIOURACIL, URACIL OR OROTIC ACID IN THE THYROID, LIVER AND SERUM OF THE RAT

Time after administration	Conc. of thiouracil, mμmoles/gm. or /ml.			Conc. of uracil, mμmoles/gm. or /ml.			Conc. of orotic acid, mμmoles/gm. or /ml.		
	Thyroid	Liver	Serum	T	L	S	T	L	S
5 minutes	96	—	—	—	—	—	—	—	—
15 minutes	128	223	198	98	22	138	0	5	0
30 minutes	146	—	—	—	—	—	—	—	—
1 hour	121	131	184	14	40	17	20	700	20
2 hours	88	—	—	8	55	16	0	40	3
5 hours	37	33	107	—	—	—	—	—	—

The data at each interval are derived from single animals. Different animals were used for the different compounds.

is no concentration gradient for thiouracil between the thyroid and serum.

It was determined that thiouracil is metabolized at a maximum rate of about 70 to 90 mμmoles per gm. of thyroid per hour. The S^{35} metabolites in the thyroid are protein-bound sulfur (PBS^{35}), thiosulfate, sulfate and an unidentified S^{35} compound. S^{35} metabolites in the liver are similar in nature to those in the thyroid except that there is less PBS^{35} formed in the liver (5 mμmoles/gm.) than in the thyroid (10 mμmoles/gm.). The majority of the S^{35} in the serum exists as free thiouracil; no significant PBS^{35} is observed.

Uptake and Metabolism of Uracil-2-C^{14} and Orotic-6-C^{14} Acid

Table 1 also reveals that the thyroid accumulates less uracil or orotic acid than thiouracil when equimolar doses (20 μmoles) are administered to rats.

Uracil metabolites.—All of the detectable C^{14} activity in the thyroid and liver exists as free uracil or as material which remains at the origin of the chromatogram and is presumed to be in nucleic acids. Forty-five percent in the thyroid and 31% in the liver is free uracil at two hours.

Orotic acid metabolites.—None of the radioactivity in the thyroid and only 10% of the C^{14} in the liver behaves as free orotic acid in one hour. The low activity in the thyroid prevented an accurate appriasal of the metabolic products.

The Relationship of the Pyrimidines to Thiouracil

The anti-thyroid action of thiouracil in the intact rat.—It is clearly evident from Table 2 that uracil does not inhibit the effects of thiouracil in: (1)

TABLE 2. THE EFFECT OF URACIL ON THE ANTI-THYROID ACTION OF THIOURACIL IN THE INTACT RAT

	Conc. of thiouracil and uracil in food %		Body wt., gm.		Thyroid wt., mg./ 100 gm.	Serum PBI^{127} μg%	I^{131} T/S ratio
			Start	End			
A	Control		93	196	6.4	3.2	29/1
B	Thiouracil	0.1	100	164	25.2	0.6	126/1
C	Thiouracil	0.1	93	143	30.8	0.8	127/1
	Uracil	0.1					
D	Thiouracil	0.1	107	162	23.5	0.6	150/1
	Uracil	0.2					
E	Thiouracil	0.1	106	169	22.2	1.1	140/1
	Uracil	0.4					
F	Thiouracil	0.1	104	162	29.2	1.1	160/1
	Uracil	0.8					
G	Uracil	0.4	100	173	7.6	3.4	34/1

6 animals in each group; values listed are averages for each group.

TABLE 3. THE EFFECT OF OROTIC ACID AND THIOURACIL ON THE FORMATION OF PBI^{131} BY SHEEP THYROID SLICES

The complete system contained sheep thyroid slices (200 mg.), 0.5 microcurie I^{131} and Krebs Ringer Bicarbonate buffer, pH 7.4, in a final volume of 3.0 ml. Incubation, 1 hour at 38°.

	Control	Control	Thiouracil	10^{-3} M	10^{-3} M	10^{-3} M	10^{-3} M	10^{-3} M	—
			Orotic acid	—	5×10^{-4} M	10^{-3} M	2×10^{-3} M	4×10^{-3} M	10^{-3} M
Uptake of I^{131} by slices, %	62*	63		66	65	70	63	68	60
PBI^{131}, % of total I^{131}	73	76		9	7	5	8	8	71

* Values are averages of duplicate flasks.

increasing the weight of the thyroid; (2) lowering the serum protein-bound iodine (PBI); or (3) increasing the "T/S ratio" of radioactive iodide. Similar negative results are obtained if orotic acid is substituted for uracil.

The inhibitory action of thiouracil on the iodination reaction in sheep thyroid slices.—Thiouracil (10^{-3} M) is very effective in inhibiting the formation of PBI^{131} by sheep thyroid slices (Table 3). Orotic acid, in doses up to 4×10^{-3} M, is not able to reverse this effect of thiouracil. Similar studies were not carried out with uracil.

The effect of uracil and orotic acid on the metabolism of thiouracil in the rat.—In these experiments, thiouracil-S^{35} (10 μmoles) and non-radioactive uracil or orotic acid are administered simultaneously to the rat intraperitoneally. At the end of an hour, the animals are killed and the tissues removed and analyzed according to methods described previously.

In Table 4, it is evident that neither uracil nor orotic acid inhibit the metabolism of thiouracil in the thyroid or in the liver of the rat.

The effect of uracil and orotic acid on the desulfuration of thiouracil by sheep thyroid cytoplasmic particles.—It was found in the present study that this thyroid cytoplasmic fraction produces a desulfuration of thiouracil *in vitro* similar to the *in vivo* metabolism in rats. The sulfur products are protein-bound sulfur (PBS^{35}), thiosulfate and sulfate.

TABLE 4. EFFECT OF URACIL OR OROTIC ACID ON THE METABOLISM OF THIOURACIL-S^{35} (10 μMOLES) BY THE THYROID OR THE LIVER OF THE INTACT RAT

	Dose pyrimidine administered, μmoles	Thiouracil metabolized, μmoles/gm./hr.	
		Thyroid	Liver
Control	—	24	30
	—	23	35
Uracil	2	16	46
	20	22	46
Orotic acid	2	23	45
	20	31	28

The data on each line are derived from single animals.

TABLE 5. THE EFFECT OF URACIL OR OROTIC ACID ON THE *in vitro* TRANSSULFURATION OF THE SULFUR OF THIOURACIL

The complete system contained the thyroid cytoplasmic particles (1.0 mg. protein), 10^{-3} M KSCN, 10^{-3} M ascorbic acid, 10^{-4} M thiouracil-S^{35}, 0.15 M Tris buffer, pH 7.4, in a final volume of 1.0 ml. Incubation 2 hours at 37°. PBS^{35} determined as in the *in vivo* experiments.

	Conc. pyrimidine (M)	PBS^{35}, mμmoles/mg. Pr.
Control	—	6.9
Control	—	6.9
	Uracil 1×10^{-4}	6.7
	5×10^{-4}	6.4
	1×10^{-3}	7.1
	4×10^{-3}	6.7
	Orotic Acid 1×10^{-4}	6.7
	5×10^{-4}	5.9
	1×10^{-3}	5.3
	4×10^{-3}	4.9

Data are derived from averages of duplicate flasks.

Uracil, in concentrations up to 4×10^{-3} M does not inhibit the transsulfuration of the sulfur of thiouracil (10^{-4} M) to the protein of thyroid cytoplasmic particles (Table 5). Furthermore, orotic acid (4×10^{-3} M) only decreases the reaction slightly—27 percent.

DISCUSSION

Thiouracil is concentrated by the thyroid in amounts greater than either uracil or orotic acid when equimolar quantities of these compounds are administered to rats. At peak concentrations, the level of thiouracil is about 1.5×10^{-4} M; that of uracil and orotic acid is about 0.98×10^{-4} M and 0.2×10^{-4} M, respectively.

From these studies, it appears that these three compounds are about equally well metabolized by thyroid tissue. However, orotic acid is metabolized in greater quantities by the liver than either thiouracil or uracil.

Sadhu (15) reported that uracil overcame the goitrogenic effect of thiouracil in rats; this was not confirmed by Paschkis *et al.* (16). Under conditions of this study, neither uracil nor orotic acid were able to reverse either the anti-thyroid effects of thiouracil in the rat or the inhibition of iodination by thiouracil in sheep thyroid slices.

Amos and Vollmayer (17) reported that thiouracil inhibited the synthesis of influenza virus by chorioallantoic membrane. Uracil was found to reverse the inhibition in these animal cells at ratios of uracil/thiouracil of 1:6 to 1:20. Similar ratios have been achieved in the present study without altering the inhibition by thiouracil.

There have been previous suggestions that thiouracil is neither incorporated into the nucleic acids of the mammalian thyroid (10, 18) nor does it inhibit the synthesis of RNA in the thyroid cell (19). The failure of uracil or orotic acid to inhibit the metabolism of thiouracil by the thyroid of the

rat or the transsulfuration of the sulfur of thiouracil to thyroid protein *in vitro* is further evidence for a non-competitive relationship between these pyrimidines and thiouracil.

It would appear that the anti-thyroid action of thiouracil does not involve a disturbance of nucleotide metabolism in mammalian thyroid tissue.

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ESTROGEN RADIOPROTECTION IN MICE[1]

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ABSTRACT

The radioprotective effect of estradiol-17β was studied using 20-day-old male Swiss mice which were injected subcutaneously with the test material, dissolved in sesame oil, once daily for the 10 days prior to an LD50 dose of Co^{60} irradiation at 30 days of age. Total doses of 10 and 20 μg of estradiol-17β afforded significant protection up to 35 days post-irradiation. Similar protection was produced by a single injection of 165 μg of estradiol-17β benzoate administered 10 days before irradiation.

IT HAS been demonstrated that estrogens exert a radioprotective effect when administered to mice prior to receiving whole-body X-irradiation (1–11). In the majority of these studies stilbestrol or estradiol benzoate was administered in one injection 5–15 days prior to irradiation, the ten-day period providing optimal results. Radioprotection by estrogens has also been demonstrated in rats (12, 13).

In this report we have confirmed the radioprotective effect of estrogens, particularly by using daily doses of estradiol administered prior to irradiation. The technic may be adapted to a method for the evaluation of the comparative radioprotective activities of various unesterified steroids.

METHODS

Male Swiss albino mice were obtained from Manor Farms, Staatsburg, New York, at 20 days of age. They received subcutaneous injections of the test compound dissolved in sesame oil (0.1 ml./injection). At 30 days of age the mice received gamma irradiation by means of a $cobalt^{60}$ source, the total dose being 665–725 r. at the rate of 17–20 r./min. (adjusted to decay of source). In any single experiment the estrogen-treated mice were exposed to precisely the same dose of irradiation as the solvent controls. The dose was determined from a half-life curve based on a previous source-strength calibration in air. During irradiation the mice were contained in a lucite cage similar to that designed by Ellinger *et al.* (14), which permits the irradiation of 12 mice simultaneously. The cage was supported by a wooden stand, the top of which was 9.2 cm. thick. The diameter of the field of irradiation at a plane through the middle of the cage compartments was 33 cm. The mice were weighed prior to irradiation and twice a week for two weeks following irradiation. The experiments were terminated 35 days after irradiation.

RESULTS

Tables 1 and 2 contain the results of experiments in which 165 μg of estradiol-17β benzoate was injected once seven and ten days respectively

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TABLE 1. PROTECTIVE ACTION OF ESTRADIOL-17β BENZOATE AGAINST Co^{60} IRRADIATION

Injected in a single dose 7 days before irradiation. Summary of 5 experiments.

Total dose (μg)	No. of mice	Number of deaths at specified days after irradiation				
		9	14	21	28	35
0	60	21	35	40	42	43
165	60	19	24*	32	35	35

* $P < 0.05$.

prior to irradiation. The ten-day interval between injection and exposure protected a significant number of irradiated mice at each of the five observation periods from 9 to 35 days after exposure, the level of significancy varying from $P < 0.05$ to $P < 0.001$ (Table 2). When the interval between treatment and irradiation was decreased to seven days, only at the 14-day post-irradiation observation was there observed a difference of limited significance in survival.

Tables 3 and 4 contain the results of experiments in which estradiol-17β was administered daily for periods of 7 and 10 days respectively prior to irradiation. The seven-day treatment period yielded some radiation protection, but in general the data were erratic. No protection was afforded with the 0.1 μg dose of estradiol-17β, whereas 1.0 μg gave significant evidence of protection at 14, 21, 28, and 35 days. The protective effect of the 10 μg dose was observed at 21, 28, and 35 days. No protection whatsoever was found when 20 μg of estradiol-17β was administered seven days before irradiation.

In contrast to the seven-day treatment period, the ten-day treatment schedule provided varying degrees of radioprotection at all of the dose levels used. When given in a dose as small as 0.1 μg, estradiol-17β provided significant protection when observations were made at 9, 14, and 21 days after irradiation, after which time significant protection could not be demonstrated. A dose of 1.0 μg likewise produced significant protection only at the 9th and 14th post-irradiation observation days. The protective effect of the 10 and 20 μg doses of estradiol-17β, however, was observed at days 9

TABLE 2. PROTECTIVE ACTION OF ESTRADIOL-17β BENZOATE AGAINST Co^{60} IRRADIATION

Injected in a single dose 10 days before irradiation. Summary of 5 experiments.

Total dose (μg)	No. of mice	Number of deaths at specified days after irradiation				
		9	14	21	28	35
0	60	16	27	38	41	41
165	60	7*	14**	22†	22‡	22‡

* $P < 0.05$.
** $P < 0.02$.
† $P < 0.01$.
‡ $P < 0.001$.

TABLE 3. PROTECTIVE ACTION OF ESTRADIOL-17β AGAINST Co^{60} IRRADIATION
Daily injection for 7 days before irradiation.

Exp. No.	Total dose (μg)	No. of mice	Number of deaths at specified days post-irradiation				
			9	14	21	28	35
A	0	12	2	3	5	5	5
	0.1	12	1	3	3	4	4
	1.0	12	2	2	2	3	3
	10	12	1	1	2	2	2
	20	12	3	4	4	4	4
B	0	12	1	4	7	7	7
	0.1	12	2	4	6	8	8
	1.0	12	0	0*	4	4	4
	10	12	2	3	3	3	3
C	0	12	3	5	7	8	8
	0.1	12	4	8	12	12	12
	1.0	12	1	2	3	3*	3*
	10	12	1	4	4	5	6
	20	12	4	5	6	7	7
A, B, C	0	36	6	12	19	20	20
	0.1	36	7	15	21	24	24
	1.0	36	3	4*	9**	10**	10**
	10	36	4	8	9**	11*	11*
A, C	0	24	5	8	12	13	13
	20	24	7	9	10	11	11

* $P < 0.05$.
** $P < 0.02$.

to 35, the significance of the 20 μg dose at 35 days being $P < 0.001$. The responses obtained with the 20 μg dose were consistent in each of the three separate experiments (Table 4). Small quantities of the estrogen (0.1 and 1.0 μg), therefore, delay the onset of radiation death without significantly affecting total mortality at 35 days. Increasing the dose of estradiol-17β to 20 μg significantly both delayed the onset of radiation death and reduced total mortality up to 35 days after irradiation.

In view of the above, the ten-day injection schedule is more satisfactory than that of seven days, for with it consistently significant radioprotection may be obtained.

Although estradiol-17β protected irradiated mice on the basis of overall survival, this treatment did not insure proper weight gain of the protected animals (Table 5). Actually, even after a total dose of 20 μg of estradiol-17β was given for a ten-day period prior to irradiation, the mean body weight of the survivors at 15 days after irradiation was essentially the same as that of the irradiated non-treated survivors.

DISCUSSION

Abrams (15) and Crosfill *et al.* (16) have shown that mice are most sensitive to radiation at 30 days of age, sensitivity decreasing with increasing

TABLE 4. PROTECTIVE ACTION OF ESTRADIOL-17β AGAINST Co^{60} IRRADIATION
Daily injections for 10 days before irradiation.

Exp. No.	Total dose (μg)	No. of mice	Number of deaths at specified days post-irradiation				
			9	14	21	28	35
A	0	12	4	5	5	5	5
	0.1	12	1	1	3	3	3
	1.0	12	0*	0**	2	2	2
	10	12	0*	1	2	2	2
	20	12	1	1	1	1	1
B	0	12	4	4	6	6	6
	0.1	12	0*	0*	0†	0†	0†
	1.0	12	3	3	3	4	4
	10	12	0*	0*	0†	0†	1*
	20	12	1	2	2	2	2
C	0	11	4	7	8	8	8
	0.1	11	2	5	6	8	8
	1.0	12	2	5	6	6	6
	10	12	2	4	5	8	8
	20	12	0*	1†	2†	2†	3*
D	0	12	2	3	3	3	3
	0.1	12	0	0	0	3	3
	1.0	12	0	2	5	5	5
	10	12	0	1	1	1	1
A, B, C, D	0	47	14	19	22	22	22
	0.1	47	3†	6†	9†	14	14
	1.0	48	5**	10*	16	17	17
	10	48	2‡	6†	8†	11**	12*
A, B, C,	0	35	12	16	19	19	19
	20	36	2†	4†	5‡	5‡	6‡

* $P < 0.05$.
** $P < 0.02$.
† $P < 0.01$.
‡ $P < 0.001$.

age. It is for this reason that mice of this age were chosen for our studies. The fact that the mice were received 10–11 days prior to irradiation should minimize the variation in sensitivity of 30-day-old mice to radiation ascribed by Crosfill *et al.* (13) to environmental change. Mice which did not gain weight properly were discarded prior to irradiation.

It can be seen from Tables 1–4 that there is no advantage to prolonging experiments such as those illustrated beyond 28 days after irradiation. In fact, for purposes of assaying compounds for their radio-protective effect where rate of animal turnover is an important factor, a 21-day post-irradiation period is suggested. Our data described here plus additional unreported data indicate that approximately 90% of those mice receiving an LD_{50} dose of radiation which are dead within 35 days will have died within 21 days.

Attempts to study compounds by using 4–12 mice per treatment group does not appear to be satisfactory. In order to obtain results which can be

TABLE 5. INFLUENCE OF IRRADIATION AND IRRADIATION AND ESTRADIOL-17β TREATMENT (10 DAYS) ON BODY WEIGHT

Total dose (μg) of estradiol-17β	Mean body wts. (gm.) at specified days after irradiation				
	0	4	8	11	15
0*	20.3 (25)†	23.4 (25)	26.2 (25)	28.4 (25)	28.7 (25)
0	21.3 (47)	18.4 (46)	19.7 (36)	20.5 (31)	22.2 (27)
0.1	21.3 (47)	18.8 (47)	20.9 (45)	21.6 (44)	22.6 (41)
1.0	21.7 (48)	18.6 (48)	20.2 (45)	21.2 (43)	20.9 (39)
10	21.1 (48)	17.8 (48)	19.6 (46)	20.9 (45)	22.7 (42)
20	20.8 (36)	17.6 (36)	19.7 (34)	20.8 (33)	22.7 (32)

* Non-irradiated, all other groups received radiation.
† Number of survivors on specified day.

TABLE 6. PROTECTIVE ACTION OF ESTROGENS AGAINST IRRADIATION WHEN ADMINISTERED TO MALE MICE

Ref.	Compound	Time of admin. prior to irrad.	Total dose, μg	No. of mice	% mortality (No. of days)
2	Control	1 Inj. 10 days	—	227	30 (40)
	Estradiol Benz.		83	40	5 (40)
	Stilbestrol		100	35	82 (40)
3 & 4	Control	Dly. inj. 7 days	—	20	100 (60)
	Estradiol Benz.		630	20	10 (60)
	Stilbestrol		700	20	80 (60)
6	Control	Combined	—	352	72 (28)
	Estradiol Benz.	1 Inj. immed. pre.	166	54	87 (28)
		1 Inj. 2 days	166	32	78 (28)
		1 Inj. 5 days	166	54	39 (28)
		1 Inj. 10 days	166	126	21 (28)
		1 Inj. 15 days	166	29	31 (28)
		1 Inj. 25 days	166	26	58 (28)
		1 Inj. 35 days	166	21	81 (28)
6	Control	1 Inj. 10 days	—	18	83 (28)
	Benzestrol		500	20	45 (28)
			1000	20	15 (28)
7	Control	1 Inj. 10 days	—	50	100 (30)
	Estradiol Benz.		166	49	37 (30)
11	Control	1 Inj. 9 days	—	68	79 (40)
	Estradiol Benz.		83	68	25 (40)
Present Study	Control	1 Inj. 10 days	—	60	68 (35)
	Estradiol Benz.		165	60	37 (35)
	Control	Dly. inj. 10 days	—	47	47 (35)
	Estradiol		10	48	25 (35)
	Control	Dly. inj. 10 days	—	35	54 (35)
	Estradiol		20	36	17 (35)

reasonably duplicated, at least 24 mice per treatment group should be used, with at least 50% of the control irradiated animals dying within 21 days. This is evident from Table 4.

Table 6 contains a summary of the results obtained by various workers. The work here reported not only confirms the radioprotective effect of estradiol-17β benzoate reported by others, but also stresses the advantage of beginning estrogen treatment ten days prior to radiation.

The procedure using 20-day-old male Swiss mice receiving daily injections for ten days prior to irradiation with cobalt^{60} provides a more sensitive measurement of the radioprotective effect of estrogens than those using one injection ten days prior to irradiation. The latter procedure requires much larger amounts of estrogen to yield suitable results.

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CHANGES IN THE THYROIDECTOMY CELLS OF THE RAT PITUITARY UNDER THE INFLUENCE OF LARGE DOSES OF TRIIODOTHYRONINE[1]

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ABSTRACT

Rats were made hypothyroid by injection of propylthiouracil (20 mg. daily) for 28 days. The pituitary changes were typical, except for the persistence of some acidophils. Then, while the rats remained on the propylthiouracil regimen, 10 μg of L-triiodothyronine sodium were injected, and animals killed 3, 12 and 24 hours later. Other rats received the same dose of T3 daily for 2, 4, 6, 8, 13 or 27 days. The aldehyde fuchsin and periodic acid-Schiff positive granulation characteristic of thyrotrophs reappeared in the thyroidectomy cells by 12 hours after triiodothyronine and disappeared again between 13 and 27 days of medication. Concomitantly with the gradual emergence of these granules, clumping and enlargement of the periodic acid-Schiff positive droplets ("T" granules of Purves and Griesbach) also occurred, and numerous angular thyrotrophs became visible, probably originating, at least in part, from "chromophobe" cells. Further, the generally fine peripheral vacuolation of the thyroidectomy cells tended to coalesce under the effect of triiodothyronine. After 27 days of triiodothyronine treatment no aldehyde fuchsin positive material was left in the pituitary, but a fair number of small thyroidectomy cells with single vacuoles persisted. These may have been irreversibly altered cells.

IN HYPOTHYROID states the aldehyde fuchsin (AF) and periodic acid-Schiff (PAS) positive angular thyrotrophs of the rat give rise to much larger, AF and PAS negative thyroidectomy cells which show progressively increasing peripheral vacuolation and the gradual emergence and accumulation of relatively large PAS+ droplets. This transformation has been discussed in detail by Purves and Griesbach (1), who also reviewed the literature concerning the thyroidectomy cell. D'Angelo (2) found that 7–10 days after withdrawal of propylthiouracil (PTU) AF+ granulation is seen in the thyroidectomy cells of rats made goitrous by this drug. Clearly, at least one of the changes that leads to the formation of these cells, degranulation, is reversible. However, the appearance of some thyroidectomy cells justifies the suspicion that they show degenerative changes (3). The purpose of this study was to ascertain how soon and to

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what extent the structure and staining properties of thyroidectomy cells of rats made goitrous with PTU change when triiodothyronine is given in a single or several large doses, and whether any evidence for possibly irreversible alterations in these cells can be obtained when hypothyroidism gives way to hyperthyroidism.

MATERIALS AND METHODS

Young adult male Sprague-Dawley rats were used. They received a diet of Purina laboratory chow *ad libitum*, and 28 daily subcutaneous (s.c.) injections of 20 mg. of PTU in aqueous suspension. In the first experiment the antithyroid regimen was continued and 10 μg of L-triiodothyronine sodium (T3)/day were injected s.c. for 2, 4, 6, 8, 13 and 27 days, at which intervals the rats were killed. Since pituitary changes were already marked after 2 days of T3 treatment, in a second experiment groups of rats that had received PTU for 28 days were killed 3, 12 and 24 hours after a single s.c. injection of 10 μg T3. In both experiments rats given PTU for 28 days but no T3 served as controls.

The number of animals per group and the effect of T3 on thyroid weight are recorded in Table 1. Only the statistical significance of thyroid weight differences with a P value <0.10 is included in the table.

All pituitaries were fixed without weighing in a mixture of SUSA fluid and saturated picric acid solution (9:1), embedded in paraffin, and cut at 4–6 μ in 3 horizontal planes. Staining procedures used in the first experiment were AF-trichrome (4), PAS with hemalum (5) and Gomori's trichrome (6). In the second experiment neither counterstaining after AF nor Gomori's trichrome procedure were applied.

RESULTS AND DISCUSSION

Control glands (Figs. 1 and 3) showed classical thyroidectomy cells largely devoid of PAS and AF+ granules, but many of which had rather fine and well scattered PAS+, AF− droplets in their cytoplasm (Fig. 1). Only an occasional normal AF+ angular thyrotroph was present, but faint AF+ staining in the Golgi region of some thyroidectomy cells could be seen

TABLE 1. THYROID WEIGHTS IN RATS TREATED WITH PTU OR PTU AND T3

Expt. No.	No. rats	Length of treatment		Thyroid wt., mg.	P (vs. control)	P (vs. next earliest treatment period)
		PTU	PTU+T3			
I	9	28 days	0	85.4±2.6*		
	9	28 days	2 days	66.4±6.4	<0.05	
	9	28 days	4 days	42.2±3.0	<0.001	
	9	28 days	6 days	40.5±1.9	<0.001	
	9	28 days	8 days	33.5±1.6	<0.001	
	9	28 days	13 days	28.7±2.2	<0.001	<0.01
	9	28 days	27 days	23.5±1.8	<0.001	<0.01†
II	7	28 days	0	78.0±3.0		
	7	28 days	3 hrs.	75.4±5.6		
	8	28 days	12 hrs.	76.9±3.4		
	8	28 days	24 hrs.	68.8±2.1	<0.05	<0.10

* Mean ± standard error.
† vs. 8 day PTU+T3 group.

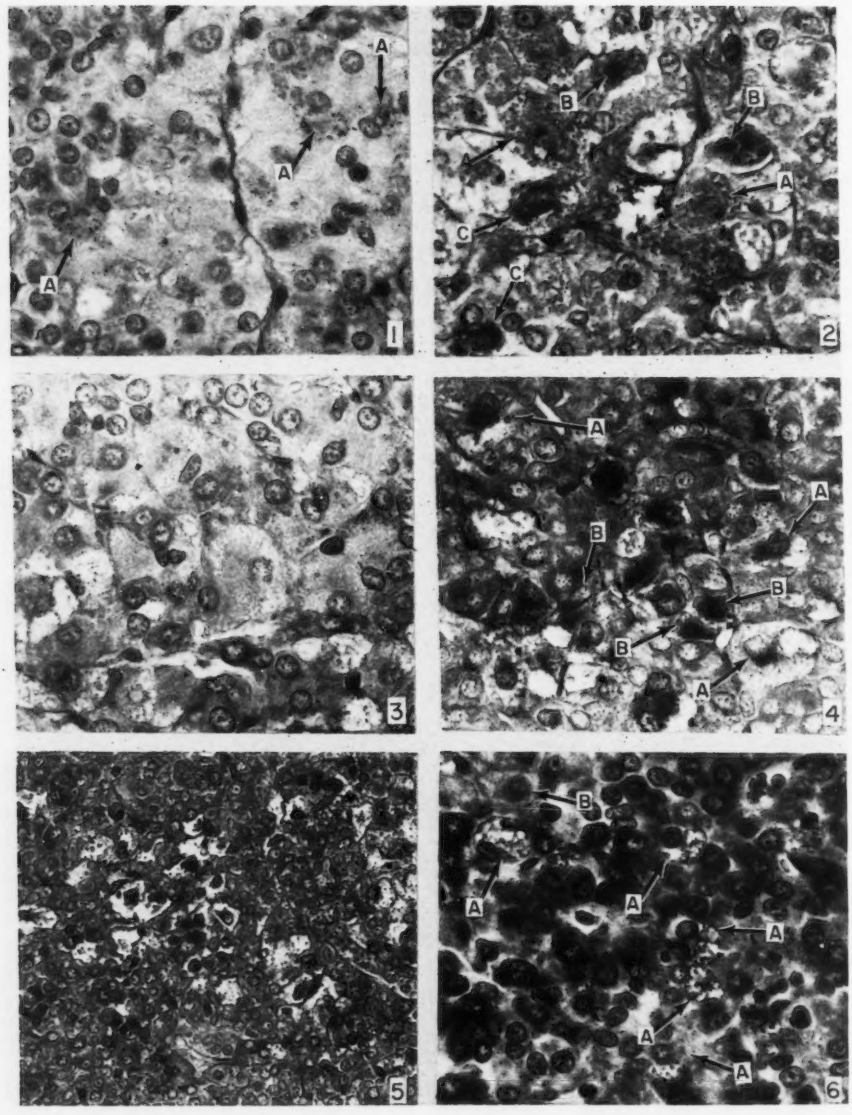

FIG. 1. Anterior pituitary lobe of control rat treated with PTU for 28 days. Some of the large, pale thyroidectomy cells show scattered, rather fine PAS+ droplets (A). Vacuolation of these cells is multiple and mostly lacy PAS, ×480.

FIG. 2. Anterior lobe of rat which received PTU for 28 days and one injection of T3 12 hours before it was killed. Thyroidectomy cells stain distinctly with PAS and tend to have larger vacuoles. PAS+ droplets are fine and scattered in some cells (A), but are large (B) in others, or show marked clumping (C). PAS, ×480.

→

when no counterstain was used. Gonadotrophs appeared normal in all control and experimental groups. In spite of the obvious goitrogenicity of the PTU treatment (Table 1), degranulation of acidophils characteristic of severe hypothyroidism was by no means complete. It is possible that the diet contained some thyroid-active substance(s) in small amounts.

Three hours after T3 many of the pituitaries could not be distinguished from those of controls when they were examined together without prior identification. In some cases, however, the changes to be described below were already recognizable.

Twelve hours after T3 the thyroidectomy cells showed unequivocal changes in most cases. *Viz.*, their granulation became distinctly AF and PAS+, their vacuoles showed a tendency to coalesce, and many PAS+ droplets were enlarged (occasionally very much so) and often clumped (Fig. 2). Further, the AF+ cells which resembled normal thyrotrophs appeared more numerous. They were sometimes massed in marginal regions of the anterior lobe where thyrotrophs are ordinarily scanty. Also, some of these cells showed a narrow band of marginal vacuolation. All these changes became more pronounced as T3 treatment was prolonged up to 8 days, and are illustrated in Figure 4 by a field from a gland of a rat that had received 8 daily injections of T3. At this late time interval, however, an additional feature shown in Figure 5 became conspicuous: the coalescence of the vacuoles of thyroidectomy cells had progressed so far that often a single space separated the granular rim of the cells from the nucleus and the adjacent granulated region. Also, 24 hours or more after the first T3 injection angular cells which were loaded with both PAS (and presumably AF)+ fine granules and coarse, clumped PAS+ droplets were sometimes present. They were similar to cells described by Purves and Griesbach (1) in rats treated with 0.1% iodothiouracil in the diet for 28 days.

FIG. 3. Anterior lobe of control rat treated with PTU for 28 days. Thyroidectomy cells appear as in Fig. 1. They show no AF+ granulation. AF-trichrome, ×480.

FIG. 4. Anterior lobe of a rat given PTU for 36 days and T3 for the last 8 days. AF+ material, occasionally in the form of coarse droplets, has reappeared (predominantly juxtanuclearly) in most thyroidectomy cells (A). AF+ cells resembling normal angular thyrotrophs (B) are also evident. In some of these cells (e.g., the one marked with B which is farthest to the right, and the unlabeled cell between A and B to the left) a narrow peripheral rim of vacuolation can be seen. AF-trichrome, ×480.

FIG. 5. Same gland as that shown in previous figure. Many throidectomy cells show confluence of vacuoles which contain little coagulated material. Juxtanuclear AF+ material is visible in most of these cells. The anterior lobe presents a Swiss cheese-like appearance which without T3 treatment occurs only in hypothyroidism of long duration. AF-trichrome, ×240.

FIG. 6. Anterior lobe of a rat treated with PTU for 55 days and T_3 during the last 27 days. Several cells which are smaller than regular thyroidectomy cells and show a single vacuole with coagulated contents are demonstrated (A). Dark cells are acidophils, cell marked with B is a gonadotroph. Gomori's trichrome, ×480.

The regranulation of the thyroidectomy cells resembled that seen by D'Angelo (2) after discontinuation of PTU treatment. In view of their early appearance, we consider it unlikely that all cells which looked like normal thyrotrophs had arisen from thyroidectomy cells. Purves and Griesbach's idea (1) that there are thyrotrophs which are so sparsely (if at all) granulated that they are classed as chromophobes may account for the emergence of AF+ thyrotrophs within a short time after T3 injection, if we assume that T3 causes the piling up of granules in such cells. The thyrotroph-like cells with peripheral vacuolation, and those which contained PAS+ droplets, on the other hand, were possibly thyroidectomy cells reverting to thyrotrophs.

The monovacuolated thyroidectomy cells depicted in Figure 5, which sometimes were so numerous as to give the pars distalis a "moth-eaten" appearance, looked like cells that were degenerating. However, we did not see any evidence of their disintegration, although after 13 days of T3 treatment their number appeared diminished.

After 13 days of T3 administration much of the AF staining of the anterior lobe had disappeared, and in the glands of rats that had received T3 for 27 days virtually no AF+ material was left. The PAS+ droplets had also diminished when T3 injections were given for 13 days, and were no longer seen after 27 days of treatment. However, cells which contained a single vacuole with abundant coagulated material in it, and whose nuclei were smaller than those of thyroidectomy cells (or even acidophils), were invariably scattered throughout the anterior lobes of rats given T3 for 27 days. Since these cells were PAS−, they could be easily distinguished from castration cells which they resembled in trichrome preparations. (No castration cells were seen in the glands examined.) It seems that these cells were altered thyroidectomy cells. Only more prolonged treatment with T_3 could show whether they were reversibly changed cells, doomed cells, or cells that persist in this form for a long time.

The disappearance of AF+ material from the hypophysis of rats subjected to prolonged T3 treatment was predictable from earlier observations (7, 8). However, diminution of acidophil size, a pituitary sign of severe hyperthyroidism (7, 9), was not seen in any of the rats in this study. The lack of extreme changes of the acidophils in our most hypothyroid control rats on the one hand and the absence of any obvious alterations in the most hyperthyroid ones on the other indicates that neither complete thyroxin deficiency nor very marked overdosage with T3 was achieved in the rats of this study. Since no staining procedure for acidophils was used for the glands of the rats killed 3–24 hrs. after T3, the earliest time of regranulation of these cells was not determined. After 2 days of T3 treatment they already appeared normal in size, number and granulation.

In conclusion, regranulation of thyroidectomy cells, and probably of "dormant" thyrotrophs, can be rapidly achieved with T3. We assume that

this is accompanied by an accumulation of TSH (2, 8). Changes in the PAS+ droplets and in the vacuolation pattern of thyroidectomy cells are also obtained. Their functional significance is obscure. Prolonged treatment with T3 eliminates all AF+ material from the pituitary of rats treated with T3 and PTU. This is probably the morphologic sign of TSH depletion (8). Vacuolated cells apparently derived from thyroidectomy cells persist during 4 weeks of T3 injections. Their ultimate fate is unknown.

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HEXOKINASE AND PHOSPHATASE ACTIVITIES OF INTESTINAL MUCOSA FOLLOWING THYROIDECTOMY AND THYROID ADMINISTRATION[1,2]

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ABSTRACT

The evidence for phosphorylation and dephosphorylation as an explanation for the "active" transfer of hexoses across the intestinal and renal epithelium is indirect and complicated by many findings that do not support this theory (1). Since the absorption of hexoses is increased significantly in hyperthyroid animals and humans and decreased in the hypothyroid state (2, 3), it was postulated that if phosphorylation and dephosphorylation were even in part responsible for hexose absorption, corresponding changes might be found in the activities of the enzymes considered to mediate hexose transfer, namely, hexokinase and phosphatase. The hexokinase activity of intestinal mucosa was found to increase significantly in thyroid-treated rats and to decrease significantly in thyroidectomized animals. The phosphatase activity of intestinal mucosa, on the other hand, did not differ significantly from that of control animals in either of these conditions. Although the hexokinase activity does change in the same direction as does glucose absorption in the hyper- and hypothyroid states and although the results appear to support the phosphorylation hypothesis, this correspondence may be fortuitous. The hexokinase activity of the intestinal epithelium may merely reflect the level of metabolic activity which provides the energy necessary for driving active transfer mechanisms.

IT HAS been known for some time that one of the factors influencing the rate of hexose absorption is the level of thyroid function (2, 3). The precise mechanism of the well documented "active" absorption of certain hexoses has not been elucidated (1). The most widely held theory is the phosphorylation-dephosphorylation of these hexoses during their transit across epithelial cells. However, no definitive evidence for this hypothesis exists and recently there has accumulated a fair body of evidence which indicates that phosphorylation is not a necessary concomitant of "active" absorption. The experiments to be reported were undertaken to determine whether or not the activity of the enzymes considered to be responsible for the "active" transport of glucose, namely, hexokinase and phosphatase, would change in the same direction as that observed for glucose absorption in the hyper- and hypothyroid states.

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METHODS AND MATERIALS

Animals: Male rats, 125 to 175 gm., were kept in individual cages for the experimental period of 3 to 4 weeks. The initial weight of each experimental rat and its control was matched as closely as possible. The thyroid-treated rats and their controls received a soft diet consisting of milk, sucrose, dog food and bread. Desiccated thyroid[3] was added to the diet of the thyroid-treated animals at a level of 30 mg. per 100 gm. food. When fed for a period of 3 to 4 weeks this diet was found to raise the metabolic rate (expressed as cc. O_2/sq. M. body surface/min. at N.T.P.) of the treated rats 68% above that of untreated controls (4). Thyroidectomy was performed according to the method outlined by Griffith and Farris (5). The thyroidectomized rats and their controls received a diet of dog chow. All animals were fed ad libitum. At the end of the experimental period the rats were anesthetized with ether and sacrificed by decapitation.

Enzyme Preparations: The whole intestine was removed and the contents flushed out by forcing cold water through the length of gut with a 50 ml. syringe. The gut was weighed after drying the outer surface and pressing excess fluid out of the lumen. The first half of the intestine was then cut open lengthwise and carefully washed in several changes of cold, distilled water. After cutting into convenient lengths, the pieces were blotted with filter paper, placed on a block of ice and the mucosa was scraped off as quickly as possible. The mucosa so obtained was mixed well and weighed aliquots were taken to prepare crude homogenates. Ten percent homogenates (W/V) were used to estimate both hexokinase and phosphatase activities. The homogenate used for determining hexokinase activity was prepared by grinding the mucosa in a mortar with 9 parts of phosphate buffer (pH 7.8) containing 0.112 M. KF. The homogenate used for the estimation of phosphatase activity was prepared in a similar way but glass-distilled water was used as the diluent.

Estimation of Hexokinase Activity: Hexokinase activity was estimated by a very slight modification of the method of Long (6). The system consisted of glucose 0.003 M; MgAc 0.005 M; KCL 0.042M; ATP 0.005M; KF 0.05 M; K phosphate buffer 0.04 M, pH 7.8. Enzyme activity was measured for a period of 6 minutes at 30° C in a total volume of 1 ml. During this time interval the activity was linear with respect to time and enzyme concentration. Appropriate controls were run at the same time. Glucose was determined on barium hydroxide-zinc sulfate filtrates (7) by the method of Nelson (8). Results are expressed as mg. glucose disappearing per mg. N per hour.

Estimation of Phosphatase Activity: The ability of the intestinal mucosa to split glucose-6-phosphate is considered to be due not to a specific enzyme but to alkaline phosphatase since the pH optimum, 8, differs from that, pH 6.5, of the specific glucose-6-phosphatase of liver and kidney (9). In this paper, phosphatase will be used to denote the activity of the intestinal mucosa toward glucose-6-phosphate. Phosphatase activity was measured for 10 minutes in a system containing final concentrations of 0.02 M sodium salt of glucose-6-phosphate (prepared from barium salt) and 0.03 M Tris buffer, pH 8, in a total volume of 0.5 ml. In preliminary experiments it was established that during this time interval the activity was proportional to both time and enzyme concentration. The reaction was terminated with trichloroacetic acid and following centrifugation, aliquots of supernatant were used to estimate the phosphate released. Appropriate controls were run at the same time. Phosphate was determined by the method of King (10). Results are expressed as the mg. P per mg. N per hour.

Nitrogen: The nitrogen content of an aliquot of each homogenate used was estimated by the microKjeldahl method (11).

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[3] The desiccated thyroid used in these experiments was obtained through the courtesy of the Eli Lilly Co., Indianapolis.

RESULTS

The results outlined in Table 1 show that following thyroid administration to normal male rats, the hexokinase activity of the intestinal mucosa, expressed as the mg. glucose disappearing per mg.N per hour, increases significantly over that of untreated controls ($P=0.05$). In thyroidectomized rats the converse is seen with respect to the hexokinase activity. There is a significant decrease in the ability of the intestinal mucosa to phosphorylate glucose when compared with that of sham-operated controls ($P<0.01$). In Table 1 it is also seen that following thyroid administration

TABLE 1. HEXOKINASE AND PHOSPHATASE ACTIVITIES OF THE INTESTINAL MUCOSA OF THYROID-TREATED AND THYROIDECTOMIZED MALE RATS

Treatment	N	Hexokinase activity (mg. glucose/mg.N/hr.)	t	P	N	Phosphatase activity (mg. P/mg.N/hr.)	t	P
Thyroid-treated	10	2.25±0.22*			14	1.46±0.12*		
Controls	10	1.74±0.12	2.10	P=0.05	14	1.24±0.06	1.62	0.1<P<0.2
Thyroidectomized	15	1.34±0.08			14	1.12±0.08		
Controls (Sham-operated)	15	1.81±0.15	2.83	P<0.01	14	1.13±0.02	0.11	0.9<P<1.0

* Mean ± Standard Error.

the activity towards glucose-6-phosphate shows a small but insignificant increase ($0.1<P<0.2$). The phosphatase activity in the mucosa of the thyroidectomized animals, however, remains at the same level as in the controls ($0.9<P<1.0$).

DISCUSSION

The results outlined above show that in thyroid-treated and in thyroidectomized rats the hexokinase activity of intestinal mucosa changes in the same direction as has been shown for glucose absorption in hyperthyroid and in hypothyroid animals and humans (2, 3). Both glucose absorption and hexokinase activity increase in the hyperthyroid state and decrease in hypothyroidism. The phosphatase activity in the mucosa, however, shows no relationship to either the level of thyroid function or to changes in glucose absorption.

Since the early work of Cori and Cori and others (1, 12, 13) there has been considerable speculation as to the possible mechanism for the selective or "active" absorption of hexoses by the intestinal epithelium. Two lines of evidence have given rise to the postulated phosphorylation-dephosphorylation mechanism: first, that the absorption of glucose is inhibited by iodoacetate and phlorizin (13) which were considered to be inhibitors of phosphorylation and secondly, that during glucose absorption there is a significant increase in the organic phosphate content of intestinal mucosa (14). This phosphate accumulation is also inhibited by phlorizin (15). The following reactions have been assumed to mediate the transfer of glucose by phosphorylation-dephosphorylation:

1. glucose + ATP $\xrightarrow{\text{hexokinase}}$ glucose-6-phosphate

2. glucose-6-phosphate $\xrightarrow{\text{phosphatase}}$ glucose + inorganic phosphate

In intestinal mucosa are found both hexokinase and an enzyme which has activity toward glucose-6-phosphate, but which is most probably alkaline phosphatase and different from the specific enzyme found in liver and in kidney (9).

A simple and attractive interpretation of the results outlined would be that the increase and decrease in hexokinase activity in hyperthyroidism and in hypothyroidism, respectively, are related in some way to the changes in hexose absorption under these conditions. Several workers claim that phosphorylation by mucosal extracts *in vitro* parallels the absorption rate of the corresponding sugar *in vivo* (16, 17, 18). Thus, a greater concentration of hexokinase, by increasing the rate of glucose phosphorylation, could increase the concentration gradient with respect to glucose between the intestinal lumen and the epithelial cells and thus could facilitate glucose transfer across the mucosa.

From the results obtained, it also appears that the hexokinase activity would be rate limiting if phosphorylation and dephosphorylation are in fact responsible for glucose absorption. Assuming that the Q_{10} for hexokinase lies between 2 and 3, the results indicate that the rate of glucose disappearance would be considerably less than the rate at which glucose-6-phosphate is split. (Glucose disappearance at 30° C: 12.3, 9.5, 7.5 and 10.1 μM/mg. N/hr. compared with glucose-6-phosphate split at 37° C: 47.1, 40.0, 37.1 and 36.5 μM/mg. N/hr.).

However, more recent evidence indicates that the postulated phosphorylation-dephosphorylation may not, in fact, be responsible for hexose absorption. Phlorizin has been shown to inhibit several enzyme systems at concentrations lower than that required to inhibit phosphorylation (19). On the basis of studies on the substrate specificity of the hexokinase of rat intestinal mucosa, Sols (20) concluded that phosphorylation by this enzyme could not be directly responsible for hexose transfer. Crane and Krane (21) have shown in everted intestinal sacs of the hamster, that the two glucose derivatives, 1-deoxyglucose and 6-deoxyglucose, which cannot be phosphorylated in the 1 and 6 positions respectively, pass from the mucosal to the serosal side at a rate almost as great as that of glucose. These workers suggest that some mechanism other than phosphorylation-dephosphorylation is responsible for the "active" transfer of glucose and its derivatives.

Another interpretation could be that the hexokinase activity merely reflects the level of metabolic activity in the cells of the intestinal mucosa. An increase or a decrease in this activity would in turn make available more or less energy which is necessary to drive "active" or uphill transfer mecha-

nisms. Thus the absorption of glucose might possibly be related only indirectly to the hexokinase activity and not necessarily to direct phosphorylation by this enzyme. Further work is necessary to determine whether such an interpretation is valid.

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HEXOKINASE AND PHOSPHATASE ACTIVITIES OF THE INTESTINAL MUCOSA IN HYPOPHYSECTOMIZED AND THYROID-TREATED HYPOPHYSECTOMIZED RATS[1]

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ABSTRACT

It has been found that in hyper- and hypothyroid states (1) the hexokinase activity of rat intestinal mucosa shows changes in the same direction as has been found for glucose absorption, namely, increased following thyroid administration and decreased following thyroidectomy (1, 2, 3). The phosphatase activity, on the other hand, showed no change in either of these conditions.

Since glucose absorption has been shown to decrease considerably following hypophysectomy (4, 5) and since this decrease has been shown to be due to thyroid deficiency (6), the hexokinase and phosphatase activities of the intestinal mucosa of hypophysectomized and thyroid-treated hypophysectomized rats were investigated. It was found that following hypophysectomy, the hexokinase activity falls significantly below that of paired-fed intact controls. Thyroid administration to hypophysectomized rats restored the hexokinase activity to the same level as their paired-fed intact controls. The phosphatase activity also showed a highly significant decrease following hypophysectomy but following thyroid administration to hypophysectomized animals, the activity of this enzyme unlike the hexokinase activity still remained significantly below that of paired-fed intact controls.

IN HYPOPHYSECTOMIZED rats glucose absorption is 30 to 35% below that of normal control animals (4, 5). Since glucose absorption is increased in the hyperthyroid state and decreased in hypothyroidism (2, 3), Russell investigated the effect of thyroxine administration on glucose absorption in the hypophysectomized rat (6). She found that thyroxine administration restores completely to normal the ability of the intestinal mucosa to absorb glucose but that it had no effect on the altered disposition of the absorbed glucose nor on the ability of the hypophysectomized rat to maintain carbohydrate stores during fasting.

The experiments to be reported were undertaken because it was thought that if phosphorylation and dephosphorylation were either directly or indirectly related to hexose absorption a decrease in the activities of the enzymes, hexokinase and phosphatase in the intestinal mucosa might ac-

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company the decreased glucose absorption in hypophysectomized animals and that the activities of these enzymes might be restored to normal in thyroid-treated hypophysectomized rats. This was found to be the case with respect to the hexokinase activity but the phosphatase activity did not fit this pattern.

METHODS AND MATERIALS

Hypophysectomized male rats 130 to 170 gm. were given a soft diet consisting of milk, sucrose, dog food and bread. In order to eliminate a decreased food intake as a possible complicating factor, intact rats were paired-fed the same amount of food the following day. The thyroid-treated hypophysectomized rats received the same diet supplemented with desiccated thyroid[2] at a level of 30 mg. per 100 gm. soft diet. Their paired-fed intact controls received the same diet without the thyroid supplement. All animals were kept in individual cages. At the end of the experimental period of 3 to 4 weeks, the rats were anesthetized with ether and sacrificed by decapitation.

Preparation of crude homogenates of intestinal mucosa and estimation of hexokinase and phosphatase activities were as described previously (1).

RESULTS

In Table 1 it is seen that following hypophysectomy the hexokinase activity of rat intestinal mucosa shows a highly significant decrease below that of paired-fed intact controls ($P < 0.001$). When desiccated thyroid is

TABLE 1. HEXOKINASE AND PHOSPHATASE ACTIVITIES OF THE INTESTINAL MUCOSA OF HYPOPHYSECTOMIZED AND THYROID-TREATED HYPOPHYSECTOMIZED MALE RATS

Treatment	N	Hexokinase activity (mg. glucose/mg.N/hr.)	t	P	N	Phosphatase activity (mg.P/mg.N/hr.)	t	P
Hyped[1]	8	1.24±0.09[3]			7	0.14±0.014		
P-F Controls[2]	8	1.68±0.11	5.8	<0.001	7	0.24±0.020	3.2	0.01<P<0.02
Hyped+Thyroid	10	1.79±0.07			11	0.16± .003		
P-F Controls	10	1.74±0.10	0.04	0.9<P<1	11	0.27± .002	5.5	<0.001

[1] Hyped = Hypophysectomized.
[2] P-F Controls = Paired-fed controls.
[3] Mean ± Standard error.

included in the diet of hypophysectomized animals, the hexokinase activity is fully restored to that of normal controls receiving the same amount of diet without thyroid ($0.8 < P < 0.9$). Table 1 also shows that in hypophysectomized rats the phosphatase activity of intestinal mucosa also shows a similar decrease ($0.01 < P < 0.02$). However, thyroid feeding had no effect on the activity of this enzyme, which remained at the low levels observed in untreated hypophysectomized rats ($P < 0.01$ compared with paired-fed intact controls).

DISCUSSION

The results reported in a previous communication (1) as well as those obtained in the present experiments show that a decreased level of thyroid

[2] The desiccated thyroid used in these experiments was obtained through the courtesy of the Eli Lilly Co., Indianapolis.

function whether induced by hypophysectomy or by thyroidectomy leads to a significant lowering of the hexokinase activity of rat intestinal mucosa. Conversely, thyroid administration to normal rats resulted in an increase in the activity of this enzyme in intestinal mucosa. When the diet of hypophysectomized animals was supplemented with desiccated thyroid, the hexokinase activity was restored to that of paired-fed intact controls. The phosphatase activity was found to bear no relationship to the level of thyroid function.

According to the phosphorylation-dephosphorylation theory of the active absorption of glucose, the hexose is phosphorylated at the luminal border of intestinal epithelial cells by the enzyme, hexokinase, and dephosphorylated at the basal end by phosphatase. The sugar enters the mesenteric blood as the intact 6-carbon unit (7). Several reports indicate that the hexokinase activity of intestinal extracts *in vitro* is adequate to account for the rate of glucose absorption *in vivo* (8, 9, 10). Sols, however, concluded that phosphorylation by this enzyme could not be directly responsible for hexose transfer (11). Other observations (12) also indicate that direct phosphorylation is not necessarily involved in the active transfer of glucose derivatives.

The experiments reported here appear to support the phosphorylation-dephosphorylation theory. However, the hexokinase activity may simply reflect the level of metabolism in the mucosal cells but may bear an indirect relationship to absorption since "active" or uphill transfers are dependent on the energy derived from cellular metabolic activity. Further work is necessary to elucidate the significance of these findings.

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INACTIVATION OF PROGESTERONE BY CELLULAR FRACTIONS OF RAT LIVER AND KIDNEY *IN VITRO*[1]

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ABSTRACT

Fresh rat liver and fresh rat kidney were rapidly minced and homogenized. Repeated refrigerated centrifugations of each homogenate, each time at higher speeds, yielded fractions which consisted, respectively, chiefly of nuclei and coarse debris, mitochondria, microsomes, and final supernatant. Each fraction as well as an aliquot of the original homogenate was incubated at 37° C with progesterone. After 60, 30, or 5 minutes the incubation was ended and the residual progestin was extracted and bio-assayed.

In general, most of the hormone had been inactivated, the liver nuclear fraction being the only one in which as much as 50–75% of the original hormonal activity consistently remained. Separate assay of the protein "bound" fraction revealed that "binding" was not a significant mechanism of inactivation. *In vitro* inactivation of progesterone by rat kidney as well as by rat liver was demonstrated.

INACTIVATION of estrogen and of progesterone *in vivo* was first demonstrated directly in the classic experiments of Zondek (1, 2, 3). He ascribed the inactivation of folliculin to the action of enzymes in the liver, and demonstrated that part of the inactivated estrogen could be released by hydrolysis. Numerous *in vivo* studies followed on hepatic conversion of sex hormones to inactive substances. Progesterone is also inactivated *in vitro* by a mince or slices of human, rat, or rabbit liver (4, 5, 6, 7, 8, 9, 10).

In vivo inactivation of progesterone by the kidney has been described in the rabbit and monkey (11, 12) but there seem to be no reports on *in vitro* inactivation of progesterone by renal tissue.

The purpose of our experiments was to investigate the inactivation of progesterone *in vitro* both by whole homogenates of rat liver and kidney and by cell fractions prepared by differential centrifugation of the homogenates.

A preliminary report has appeared (13).

MATERIALS AND METHODS

Liver was quickly removed after death from adult female rats and was fractionated according to the method of Hogeboom (14). Approximately 1 gm. of hepatic tissue was weighed, then immersed in 0.25 M sucrose solution (9 ml. to 1 gm. tissue) at 0° C. The

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tissue was immediately minced with scissors, then homogenized rapidly in a tissue grinder with a Teflon pestle for two minutes.

Ten ml. of the homogenate were carefully placed as a layer over 10 ml. of 0.34 M sucrose solution in a 30 ml. test tube. The liquid was centrifuged for 10 minutes at 700×g and about 5° C in an International refrigerated centrifuge. Examination by phase microscopy showed that the resulting precipitate, the "nuclear fraction" (Table 1), contained nuclei, erythrocytes, connective tissue, and residual intact cells.

The supernatant was centrifuged for 10 minutes at about 5° C and 5,000×g, resulting in an opalescent supernatant, a pink, partially sedimented layer of particles, and a pellet of mitochondria. The pellet was removed quantitatively with 0.25 M sucrose solution and redispersed in 5 ml. 0.25 M sucrose solution in a tube which was then centrifuged for 10

TABLE 1. INACTIVATION OF PROGESTERONE BY CELL FRACTIONS

	With DPN and nicotinamide			Without DPN and nicotinamide	
Rat	#4	#5	#5	#6	#7
Incubation in minutes	60	60	30	30	5
Liver					
Total homogenate	74%		99%	77%	99%
Nuclear	43		50	24	27
Mitochondrial	98		99	99	98
Microsomal	98		99	95	99
Final supernatant	98		99	41	99
Control					< 3
Kidney					
Total homogenate		99%		67%	27%
Nuclear		99		89	99
Mitochondrial		77		99	98
Microsomal		98		99	99
Final supernatant		89		32	99
Control					< 3

minutes at 24,000×g and about 5° C. The resultant precipitate was suspended in sucrose solution and was again centrifuged for 10 minutes at 24,000×g and about 5° C. The final precipitate, the "mitochondrial fraction," was reasonably pure cytologically on microscopic examination.

All supernatants from the preparation of the mitochondrial fraction were combined and were made up to 35 ml. with 0.25 M sucrose solution. The resulting liquid was placed in tubes $\frac{5}{8}$ in. in diameter and was subjected to the maximal speed of the centrifuge for two hours at about 5° C. This yielded a "final supernatant" and a precipitate in the form of a firmly packed, reddish, transparent pellet. The latter was resuspended in 0.25 M sucrose solution as the "microsomal fraction."

Whole homogenates and cellular fractions of fresh kidneys from the rats which had supplied liver tissue were prepared in identical fashion.

Incubations were carried out in general according to the method of Taylor (7). To a 1 ml. aliquot of each preparation were added 1 ml. of phosphate-saline buffer containing 1 mM sodium citrate, 1 ml. of phosphate-saline buffer at pH 7.4, and 0.5 mg. crystalline progesterone dissolved in 0.05 ml. sesame oil. In addition, 0.7 mM of diphosphopyridine nucleotide (DPN) and 40 mM nicotinamide were added as co-factors in some cases (Table 1). Each mixture was incubated with frequent shaking at 37° C for 5, 30, or 60 minutes. Then the reaction was stopped by adding 5 ml. cold acetone. A control specimen was prepared by the addition of cold acetone at once and without incubation to an aliquot of one of the fractions.

The homogenates were well stirred in the acetone, yielding a flocculent precipitate. The latter was three times re-suspended in cold acetone and ether, the suspension each time being re-centrifuged. The final precipitate was considered to contain the protein "bound" fraction of progestin, whereas the "free" fraction of progestin was dissolved in the supernatants. The latter were pooled, and a measured volume of sesame oil was added. The acetone and ether were removed by evaporation and distillation, leaving an emulsion composed of oil (in which were dissolved the acetone- and ether-soluble substances in the homogenate) and the aqueous residue of the original preparation. Oil and water phases were separated by centrifugation, and the oil was set aside for bio-assay of the "free" progestin.

The fraction of the homogenate which had precipitated in acetone and ether was re-suspended. The pH was adjusted to about 1 with hydrochloric acid. The mixture was then hydrolyzed for six hours in a boiling water bath to release the protein "bound" progestin. The hydrolysate was extracted five times with ether. Then, as with the "free" fraction, sesame oil was added, the ether was removed, the aqueous residue was separated, and the oily solution was ready for assay for "bound" progestin.

The bio-assay was conducted with CHI mice according to a standard technique (15). The bio-assay, originally considered specific for progesterone, is now known to detect Δ^4-3-ketopregnene-20α-ol and Δ^4-3-ketopregnene-20β-ol, and possibly other naturally occurring progestins as well (16). Assay results are expressed in Progesterone Equivalents; one Progesterone Equivalent is defined as equal to the *activity* of 1 mg. of progesterone when the latter is assayed by the procedure of Hooker and Forbes in the absence of estrogen and other interfering factors (17).

RESULTS

The final concentration of protein "free" progestin, expressed as Progesterone Equivalents, as determined by bio-assay of the non-incubated control preparations and of each incubated preparation was used to calculate the percent loss of activity. The results appear in Table 1. It is evident that in most preparations inactivation was far advanced or complete.

The protein "bound" fraction of progestin (not represented in Table 1) in all cases contained less than 1% of the original activity of the hormone.

DISCUSSION

In vitro inactivation of progesterone by renal tissue is clearly demonstrated.

Comparison of the various values for a given preparation (Table 1) shows either that, as for the liver mitochondrial fraction, inactivation was complete in all cases or that, as for the total liver homogenate, the residual activity varied considerably. Such variation cannot be consistently related either to the length of incubation or to the presence or absence of co-factors, but perhaps is due largely to biologic differences between donor rats. However, inactivation was less than 51% in all liver nuclear fractions, while the kidney nuclear fraction, the liver mitochondrial fractions, and the microsomal fractions of both organs consistently showed ability for inactivation of most or all the progesterone. Consequently, it may be postulated that the inactivating agent, believed by Zondek (1, 2), Taylor (7), and others to be an enzyme or enzymes, is associated in considerable extent with the

mitochondria and microsomes of liver and kidney cells. At the same time it is evident that the inactivating agent was present to some degree in every incubated fraction as prepared.

Earlier experiments (18) have shown that acid hydrolysis to release progestin "bound" to protein appears not to destroy the progestin. Since in the present studies little if any progestin was found in association with the acetone- and ether-insoluble fractions after their hydrolysis, it must be inferred that protein "binding" was not a significant factor in either hepatic or renal inactivation *in vitro*. This finding agrees with earlier observations that inactivation of endogenous progesterone *in vivo* during renal passage in the rabbit and monkey is not due in significant degree to "binding" (12), but is in contrast with the observation that exogenous progesterone is "bound" *in vivo* by the liver of the mouse (19).

It is a limitation of the bio-assay that it does not identify compounds but only distinguishes between those which do and those which do not evoke the same response as does progesterone in the test animal. The residual activity in the liver and kidney preparations could have been due to progesterone, perhaps to its naturally occurring metabolites, the α and β isomers of Δ^4-3-ketopregnene-20-ol, or possibly to another, unidentified compound which duplicates the progesterone effect in the test mouse. Since inactivation was not due to "binding" to protein, it presumably was the result of transformation of progesterone to inactive steroids, but identification of the latter is not possible by bio-assay.

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THE EFFECTS OF STEROID HORMONES UPON AMINO ACID INCORPORATION INTO MOUSE KIDNEY HOMOGENATES[1]

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ABSTRACT

The effects of a number of androgenic and adrenal-cortical steroids upon the steroid-sensitive amino acid incorporating system of mouse kidney homogenate have been explored. The responses to methylandrostenediol, androsterone, methyltestosterone, and testosterone were similar to that observed previously to testosterone propionate, namely, an increase in incorporation rate which reaches a maximum (25–35% above that for control) within two to four days, followed by a rapid decline to the pre-stimulatory rate. Desoxycorticosterone produces a somewhat smaller increase after two days, while treatment with hydrocortisone results in a decrease of 30–35%.

Hydrocortisone, in adequate amounts, inhibits the response to testosterone propionate almost completely, while the inhibitory effect of the former is not diminished by relatively large amounts of the latter. It is concluded that the two compounds exert their effects at different metabolic sites.

IT HAS been shown that injection of testosterone propionate (TP) in A Cloudman mice is followed by an increase in the rate of incorporation of labelled glycine into kidney protein *in vitro*. Thus, in mice given 1.0 mg. TP daily for 2 days the rate of incorporation of glycine-1-C^{14} into the proteins of kidney slices (1) or homogenates (2) was increased 30–50%, whereas there was no effect upon incorporation into diaphragm protein.

A number of other steroids have been examined for their effect upon the incorporation of labelled glycine into proteins of mouse kidney homogenates. The compounds used were testosterone, methyl testosterone, methyl-androstenediol, androsterone, hydrocortisone, and desoxycorticosterone.

In addition, the interaction of testosterone propionate and hydrocortisone, in their effects upon amino acid incorporation, has been studied.

EXPERIMENTAL

Female A Cloudman (Ajax) mice,[2] 3–5 months of age, were used. The several steroids were injected subcutaneously as solutions (or suspensions) in sesame oil containing 10 mg./ml. One-tenth ml. was injected once daily

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[2] Obtained from Jackson Laboratories, Bar Harbor, Maine.

for one to six days. Six to eight animals were included in each group, and a similar group of uninjected animals served as a control for each experiment (1). Eighteen to 24 hours after the last injection, the mice were killed and each kidney was homogenized separately in nine times its weight of Krebs-Ringer phosphate buffer containing 0.2 μC/ml. of glycine-C^{14}. The total glycine concentration was 0.5 mM. The homogenates were incubated for 0.5 hour at 39.5°, the reaction stopped by the addition of trichloroacetic acid to 5%, and the labeled protein isolated as described previously, and counted with a thin-window flow counter. The data were corrected for self absorption to a uniform sample thickness of 5 mg./cm^2. Under these condi-

TABLE 1. EFFECT OF METHYL ANDROSTENE DIOL UPON INCORPORATION OF GLYCINE-1-C^{14} INTO MOUSE KIDNEY HOMOGENATE

Group No.	No. of animals	Duration of treatment (1.0 mg. MAD/day)	Incorporation rate* ($\times 10^2$)	Percent increase†
A	6	—	4.62 ± .32	—
B	6	2 days	6.05 ± .54	31
C	6	4 days	5.87 ± .50	28
D	6	6 days	4.58 ± .34	− 1

* Incorporation rate = μM glycine carbon incorporated per gram protein per hour. Ave ± S.E.

† Compared to uninjected controls.

tions, the specific activity of the protein isolated from homogenates prepared from the kidneys of untreated animals averaged about 2.2 ± 0.17 cpm/mg.; this corresponds to an incorporation rate of 0.042 micromole of glycine per gram protein per hour. The standard error of the mean for each group was usually from 5–10% of the mean; differences between groups of the order of 20% were therefore considered to be highly significant ($P < 0.01$).

RESULTS

In Table 1 are summarized the data obtained for groups of mice treated with methylandrostenediol for 2, 4, and 6 days, respectively. Data for TP, testosterone (free alcohol), methyl testosterone, and androsterone are indicated graphically in Figure 1.

All of the androgens show a characteristic pattern—a gradual increase during 2–4 days to a rate which is 30–40% (65% for TP) above the rate for homogenates prepared from kidneys of untreated animals, followed by a wearing-off phase such that the control value is restored after 6 days.

Data for hydrocortisone and desoxycorticosterone are illustrated in Figure 2. Hydrocortisone, as might be expected, inhibits glycine incorporation, with no indication of a tendency to return to normal during four days of continuous treatment. The pattern for desoxycorticosterone is similar to that displayed by the androgenic steroids, except that a somewhat lower maximum is reached after but one day.

Some experiments were carried out to determine whether or not any of the compounds studied would affect incorporation rates when added *in vitro*. When kidney slices from untreated animals were incubated in buffer containing added steroids at concentrations of 0.0035–0.035 mM, no effect

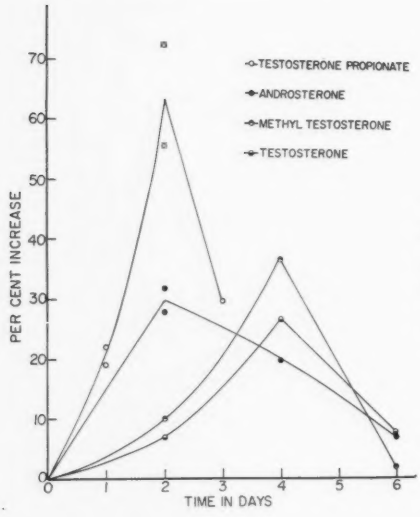

FIG. 1. Effects of androgenic steroids upon incorporation of glycine-1-C^{14} into protein of mouse kidney homogenate. Abscissa: time in days after beginning injections (1 mg./day). Ordinate: percent increase in incorporation rate compared to untreated animals.

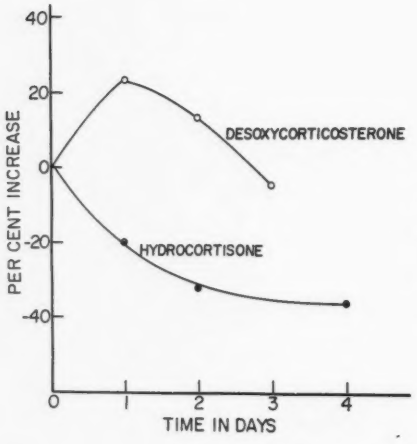

FIG. 2. Effects of desoxycorticosterone and hydrocortisone (1 mg./day) upon incorporation of glycine-1-C^{14} into proteins of mouse kidney homogenate. Abscissa: time in days after beginning injections. Ordinate: percent increase (or decrease) in incorporation rate compared to untreated animals.

upon the incorporation of labelled glycine could be observed for any of the compounds tested. At higher steroid concentrations significant inhibition was consistently observed with each compound. The most inhibitory in this respect was desoxycorticosterone, which reduced incorporation by 95% when its concentration was 0.5 mM.

TABLE 2. EFFECTS OF TESTOSTERONE PROPIONATE AND HYDROCORTISONE UPON INCORPORATION OF LEUCINE-1-C^{14}*

Steroid used	Dose, mg./day	No. of animals	Incorporation rate† ($\times 10^2$)	Percent change
Testosterone propionate	0.0	8	12.7 ± 1.4	
	0.10	8	15.1 ± 2.0	+19%
	0.33	8	19.1 ± 2.6	+50%
	3.0	8	18.5 ± 1.2	+46%
Hydrocortisone	0.0	8	7.4 ± 0.2	
	0.5	8	6.8 ± 0.6	− 8
	1.0	7	4.8 ± 0.2	−35
	2.0	8	5.2 ± 0.24	−30

* DL-leucine-1-C^{14}, 0.73 mM, 0.2 μC/ml.
† Incorporation rate = μM leucine carbon incorporated per gram protein per hour. Ave. ± S.E.

The observation that TP served to increase the rate of amino acid incorporation, while hydrocortisone had the opposite effect, prompted us to study the quantitative aspects of the effects of these two compounds, singly and in combination. For these experiments, labelled leucine was used after appropriate experiments had shown that the incorporation of leucine, like that of glycine, was increased by TP and inhibited by hydrocortisone.

Table 2 shows that as little as 0.1 mg. of TP daily for two days causes a significant (19%) increase in incorporation rate, and that stimulation is maximal with 0.33 mg./day. Only slight inhibition occurred with 0.5 mg. of hydrocortisone, and maximum inhibition occurred with 1.0 mg. daily.

Table 3 summarizes the results which were obtained when the response to increasing amounts of TP was measured in animals which were simultaneously receiving hydrocortisone. Comparison of the data of Table 3 with those in Table 2 reveals that under these conditions the response to TP is not diminished by 0.5 mg. of hydrocortisone; but is nearly completely abolished by 1.0 mg. of hydrocortisone daily. In contrast, the inhibitory effect of hydrocortisone is still evident in the presence of 3 mg. daily of TP (last two lines of Table 3).

TABLE 3. EFFECTS OF SIMULTANEOUS ADMINISTRATION OF TESTOSTERONE PROPIONATE AND HYDROCORTISONE UPON INCORPORATION OF LEUCINE-1-C^{14}*

Testosterone, mg./day	Hydrocortisone, mg./day	No. of animals	Incorporation rate† ($\times 10^2$)	Percent change
0	0.5	6	6.4 ± 0.3	—
0.10	0.5	6	8.4 ± 0.4	+31
0.50	0.5	6	10.4 ± 1.0	+62
3.0	0.5	6	10.2 ± 0.8	+60
0	1.0	6	9.4 ± 0.5	—
0.10	1.0	6	10.2 ± 0.7	+ 8
3.0	1.0	6	11.3 ± 1.0	+21
3.0	0.0	6	17.3 ± 1.2	—

* DL-leucine-1-C^{14}, 0.73 mM, 0.2 μC/ml.
† Incorporation rate = μM leucine carbon incorporated per gram protein per hour. Ave. ± S.E.

DISCUSSION

Although the effects of androgenic steroids upon various aspects of protein metabolism in intact animals have been explored in considerable detail (3), there are no reports of studies dealing with the effects of these compounds upon amino acid incorporation *in vitro* by tissues other than those of the reproductive tract. The data reported here indicate that the property of inducing a transitory increase in the rate of incorporation of glycine into mouse kidney protein is shared by a number of androgenic steroids. In our experiments, the increases observed for testosterone propionate and methyl testosterone were larger than those observed for the other compounds studied; these differences, however, do not necessarily reflect the relative anabolic potencies of the various compounds since no effort was made to determine the minimal effective dose for any androgen except testosterone propionate.

Inhibition of amino acid incorporation into proteins of rat diaphragm following administration of adrenocortical steroids has been reported by Wool and Weinshelbaum (4) and by Manchester (5). There is evidence, too, for an inhibitory effect of cortical steroids upon uptake of amino acids *in vivo* (6, 7). The data of Table 3 indicate that besides its inhibitory effect upon incorporation of amino acids into kidney protein, hydrocortisone also antagonizes the action of testosterone propionate. The non-competitive nature of the inhibition, as indicated by the fact that 1 mg. of hydrocortisone nearly completely reversed the stimulatory effect of 3 mg. testosterone propionate while half this dose had no effect upon 0.1 mg. of the androgen, together with the lack of a reciprocal antagonism by testosterone propionate, suggests that the two compounds exert their effects at different metabolic loci.

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THE INFLUENCE OF HYPOPHYSECTOMY AND OF GROWTH HORMONE ADMINISTRATION ON THE OXIDATION OF PALMITATE-1-C^{14} BY THE UNANESTHETIZED RAT[1]

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ABSTRACT

The conversion of intravenously administered albumin-bound palmitate-1-C^{14} to expired $C^{14}O_2$ by unanesthetized rats was used as an index of fatty acid oxidation. It was found that hypophysectomy did not influence the oxidation of palmitate to CO_2 whether this was determined in the fasted or fed state. Bovine growth hormone treatment, chronic or acute, did not appear to enhance the utilization of palmitate by fed or fasted hypophysectomized rats. These findings, along with the evidence of others, suggest that the utilization of fatty acids is under the regulation of factors other than growth hormone.

ONE of the more dramatic sequels of growth hormone administration in a variety of mammals is a rapid mobilization of lipid from adipose tissue stores (cf. 1, 2, 3, 4, 5, 6). While a large body of indirect evidence (1, 2, 7, 8) has led to the suggestion that growth hormone also has the property of enhancing fatty acid oxidation by hepatic and extrahepatic tissues, direct evidence for this view has not been forthcoming. The present study was undertaken to determine the effects of hypophysectomy and of growth hormone administration on the oxidation of albumin-bound palmitate-1-C^{14} by the unanesthetized rat.

MATERIALS AND METHODS

The palmitic acid-1-C^{14} was prepared for injection by the method of Bragdon and Gordon (9). One ml. of the resultant palmitate-rat serum mixture (0.9 μc/59 μg palmitate/ml.) was injected into the tail vein of rats restrained in a towel.

All animals were adult females (Charles River strain) weighing 200 ± 30 gm. Hypophysectomies were performed 3–16 weeks before initiation of the experiments.

Bovine growth hormone (N.I.H.-BGH-1)[3] was administered subcutaneously as a single injection (3 mg./rat) 5 hours before the palmitate injection (acute experiments) or in 4 daily injections (3 mg./rat/day), the last being given 12 hours before the palmitate injection (chronic experiments). All animals in the latter group exhibited a highly significant weight gain in response to treatment.

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[2] Postdoctoral Fellow of the National Heart Institute.

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All animals were maintained on Purina laboratory chow prior to experiment. In addition, the "fed" animals were given 2 ml. of a 50% glucose solution by stomach tube 1 hour before the palmitate injection. The "fasted" animals were given this glucose feeding 24 hours before the palmitate injection and were not permitted access to food thereafter.

Immediately following the palmitate injection the rats were placed in a metabolism cage through which CO_2 free air was drawn at a rate of approximately 0.7 L/minute into graduated cylinders containing 300 ml. of 3N NaOH (10). The expired CO_2 was precipitated with $BaCl_2$ from suitable aliquots of the NaOH solution obtained at intervals during the collection period. The $BaCO_3$ was centrifuged and washed successively with water, absolute alcohol and acetone. The resulting suspension was transferred quantitatively onto a tared filter paper disc and filtered with the aid of a vacuum filter tower. The discs were weighed, placed in aluminum planchets and counted in a thin window, gas flow counter (Nuclear-Chicago). Counts were corrected for background and self-absorption. Aliquots of the original albumin-bound palmitate solution were diluted with saline, plated at infinite thinness, and counted in like manner.

Non-esterified or free fatty acid (FFA) concentration in the plasma was determined by the method of Dole (11) in a separate series of animals under experimental conditions identical to the above at times corresponding to palmitate injection (time 0 of the experiments).

RESULTS

Effect of hypophysectomy

The effects of fasting and carbohydrate prefeeding on the conversion of palmitate to CO_2 by normal, intact rats is illustrated in Figure 1. Hypophysectomy did not significantly alter the pattern of palmitate oxidation in

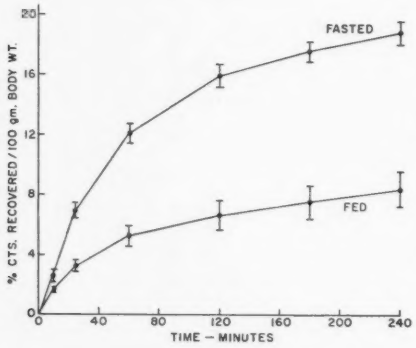

FIG. 1. Influence of fasting and glucose feeding on the cumulative recovery of intravenously administered palmitate-1-C^{14} as expired $C^{14}O_2$ in normal rats. Each point represents the mean of 3 to 7 observations in as many animals. Vertical lines: standard errors of means.

either the fed or fasted state during the four hour collection period (Figs. 2 and 4). The cumulative $C^{14}O_2$ excretion curves of normal and untreated hypophysectomized animals are essentially identical.

Effect of growth hormone administration

Chronic treatment. The administration of growth hormone (3 mg./rat/day for 4 days) did not significantly change the conversion of palmitate to CO_2 in either the fed or fasted state (Fig. 2). Total CO_2 output during the four

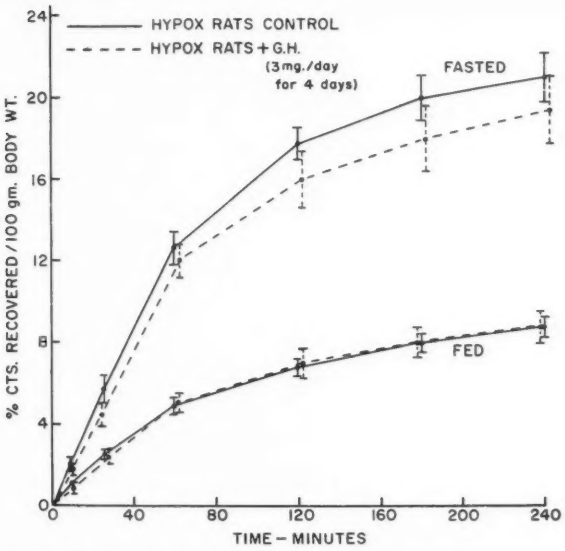

FIG. 2. Effect of growth hormone (chronic treatment) on the cumulative recovery of palmitate-1-C^{14} as expired $C^{14}O_2$ in hypophysectomized rats. Each point is the mean of 3 to 10 observations in as many animals. Vertical lines: S.E. of means.

hour collection period was similarly unchanged by the growth hormone regimen. The specific activity of the expired CO_2 of the growth hormone treated animals did not differ significantly from that of the hypophysectomized controls (Fig. 3).

The chronic growth hormone treatment did not result in a statistically significant elevation in plasma FFA concentration when measured 12 hours after the last injection (Table 1).

Acute treatment. The administration of a single dose of growth hormone (3 mg./rat) 5 hours before the palmitate injection significantly *decreased* the recovery of $C^{14}O_2$ in the fasted animals but was without effect in the glucose fed rats (Fig. 4). This effect of growth hormone was reflected in a significant reduction in the specific activity of the expired CO_2 during the

TABLE 1. EFFECT OF GROWTH HORMONE ADMINISTRATION ON THE PLASMA FFA CONCENTRATION OF HYPOPHYSECTOMIZED RATS

Each value represents the mean ±S.E. of 3 to 5 observations.

Animals	Plasma FFA concentration (μeq./100 ml.)		
	Control	Acute G.H.	Chronic G.H.
Fed	8.2±0.4	15.5±3.7	7.9±0.5
Fasted	38.4±2.0	63.4±5.5*	41.8±3.8

* Significantly different from control at 1% level.

first 90 minutes of collection in the fasted rats (Fig. 5). Total CO_2 output remained unchanged.

Acute growth hormone treatment significantly elevated plasma FFA concentration in the fasted animals but not in the fed rats (Table 1).

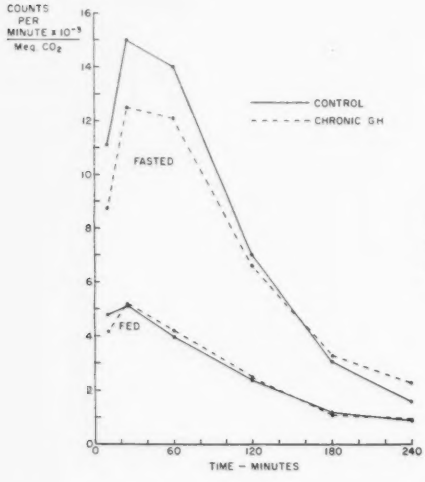

FIG. 3. Specific activity of expired CO_2 recovered at varying time intervals following injection of palmitate-1-C^{14} into the animals shown in Figure 2.

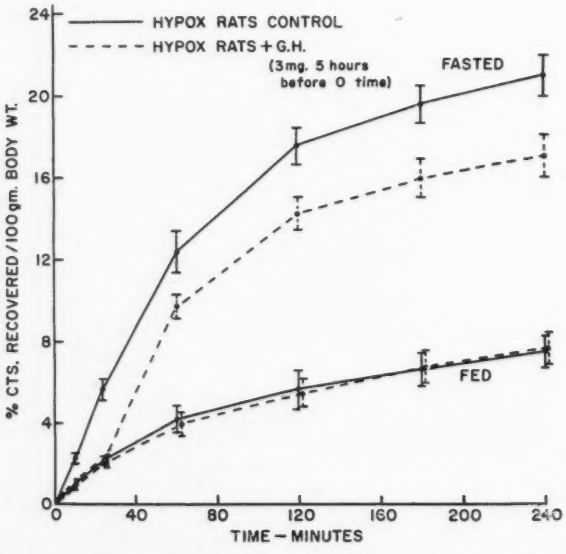

FIG. 4. Effect of growth hormone (single injection) on the cumulative recovery of palmitate-1-C^{14} as expired $C^{14}O_2$ in hypophysectomized rats. Each point is the mean of 4 to 9 observations in as many animals.

DISCUSSION

These studies indicate that the absence of the pituitary gland and hence growth hormone deprivation does not influence the rate of palmitate-1-C^{14} utilization *in vivo* as evidence by $C^{14}O_2$ output. This finding is in accord with earlier studies in which it was found that the utilization of administered C^{14}-labeled octanoate and trilaurin by hypophysectomized rats did not differ from normal if the experiments were performed a short time following the operation (12). Prolonged hypophyseal deprivation did result in a small reduction in fatty acid oxidation but this was returned to normal by thyroxine therapy suggesting that the absence of growth hormone was

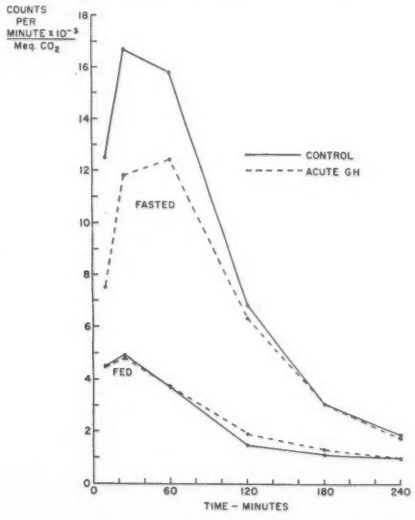

FIG. 5. Specific activity of expired CO_2 in in experiments depicted in Figure 4.

not a factor in this observation (12). Similarly, Matthews *et al.* (13) observed that hypophysectomized and normal rats oxidized equal quantities of tripalmitin to CO_2. It has further been shown by Chaikoff and his associates (14, 15) that liver slices from hypophysectomized rats oxidize acetate to CO_2 at a normal rate. Preliminary experiments in this laboratory (16) have failed to reveal a significant difference between the ability of diaphragms from normal and hypophysectomized rats to oxidize albumin-bound palmitate. The sum of the above observations leads to the conclusion that a reduction in circulating growth hormone does not interfere with the processes associated with the complete oxidation of fatty acids to CO_2.

The effects of growth hormone administration on fatty acid utilization are difficult to interpret. In the present study, growth hormone did not alter the oxidation of administered palmitate by fed hypophysectomized rats. In such animals, growth hormone did not increase the plasma FFA concentration. When growth hormone increased plasma FFA concentration,

the recovery of $C^{14}O_2$ from palmitate-1-C^{14} and the specific activity of the expired CO_2 were significantly reduced. This phenomenon can best be explained by the dilution of the administered palmitate by endogenous fatty acids mobilized in response to growth hormone administration. Increased carbohydrate oxidation by the fasted animals in response to acute growth hormone treatment could also account for this observation (cf. 17). The complexities of the *in vivo* system and the lack of detailed information concerning the effects of growth hormone on pool size and turnover rates of fatty acids prohibit definitive conclusions, but the data do suggest that under the experimental conditions employed, growth hormone administration does not appear to increase palmitate oxidation to CO_2. This view is in accord with the observations of others that growth hormone treatment (*in vivo* or *in vitro*) does not accelerate the oxidation of palmitate or acetate by rat liver slices (18, 19, 20) and with our own recent finding that growth hormone, *in vivo* or *in vitro*, does not influence the oxidation of albumin-bound palmitate by the isolated diaphragms of hypophysectomized rats (16). It would seem, therefore, that while growth hormone has a profound effect on fatty acid mobilization, the cellular utilization of the substrate thus made available may be under the regulation of other factors.

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NOTES AND COMMENTS

THE EFFECT OF LACTATION ON PLASMA LH

ABSTRACT

Lactation prevented the elevation in plasma LH activity which occurred in non-lactating, ovariectomized, female rats.

Considerable evidence indicates that lactation in addition to augmenting the secretion of luteotrophin by the adenohypophysis inhibits the secretion of the other gonadotrophins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). Thus, Desclin (1) has demonstrated that lactation prevents the formation of castration cells in the pituitary of ovariectomized rats. Recently, Rothchild and his collaborators (2, 3) have made an extensive study of gonadotrophin secretion in lactating rats using such indices of their secretion as compensatory hypertrophy of a remaining ovary and growth of ovarian grafts. The results indicated a suppression of FSH-LH release but did not distinguish between these two gonadotrophins. Parlow and Rothchild (4) have since reported that lactation decreased the LH content of the pituitary in both intact and castrated females. In order to ascertain whether release of LH was also inhibited by lactation, we have measured plasma LH in lactating rats.

METHODS

All rats were of the Sherman strain. Ovariectomized donors were used since the ovarian ascorbic acid assay for LH is not sufficiently sensitive to measure plasma LH in intact rats, and since the use of spayed animals eliminates ovarian steroids, such as progesterone, which might secondarily inhibit LH secretion during lactation.

Three groups of ovariectomized rats (numbered 1–3 as in Table 1) served as donors of plasma for the assay of LH. Group one consisted of adult virgin females which were ovariectomized while anesthetized with ether. Group two was composed of rats which were ovariectomized on the second day of lactation and had their litters removed. Donor rats of group three were ovariectomized on the second day of lactation and returned to their litters, which were adjusted to contain 8 infants per litter. Only dams which maintained their weight and whose litters grew well were used. Two weeks following ovariectomy, all three groups of rats were anesthetized with ether, and 6–8 ml. of blood were withdrawn from the jugular vein

TABLE 1. THE EFFECT OF LACTATION ON THE PLASMA LH OF OVARIECTOMIZED RATS

Type of donor rat	No. of donor rats	No. of assay rats	Percentage ovarian ascorbic acid depletion in assay rats
1. Ovariectomized			
Pool No. 1	2	3	12.9
Pool No. 2	2	3	15.7
Pool No. 3	3	4	7.2
Mean	7	10	11.4 ± 2.2
2. Ovariectomized—litters removed	6	9	13.5 ± 2.2
3. Ovariectomized—lactating			
Pool No. 1	6	9	2.6 ± 1.4
Pool No. 2	4	6	4.4 ± 2.0
Mean	10	15	3.3 ± 1.1

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over a period of 1–3 minutes into a heparinized syringe. After centrifugation, the pooled plasma from each group of rats was immediately assayed for LH activity using the ovarian ascorbic acid technique of Parlow (5) as employed in this laboratory (6). One to three pools of plasma were assayed for each group of donor rats as indicated in Table 1. Since no significant difference in results between pools of plasma from same type of donor rat was found, the arithmetic mean result is also presented in Table 1.

For assay of LH activity, one ovary of each assay rat was first removed for a control value of ovarian ascorbic acid. Then 2 ml. of plasma were injected intravenously over a period of 45–60 seconds. One hour later the second ovary was removed, and its ascorbic acid concentration determined. The results have been expressed as the percentage depletion (decrease) in ascorbic acid concentration of the second ovary as compared to the control value obtained in the first gland.

RESULTS AND DISCUSSION

The results presented in Table 1 demonstrate that a significant ovarian ascorbic acid depletion occurred in the assay rats after injection of plasma from ovariectomized rats or ovariectomized rats whose litters were removed ($P < 0.001$ for each group). In contrast to these two control groups the plasma from ovariectomized, lactating rats induced only a minimal ovarian ascorbic acid depletion, which was significantly less than the ascorbic acid depletion obtained with plasma from either of the two control groups ($P < 0.001$ on comparison with either control group).

Since ovarian ascorbic acid depletion has been shown to be a specific assay for LH under the conditions employed (5, 6), it is clear that lactation prevents the rise in plasma LH-activity which normally follows ovariectomy. If, as appears highly likely, plasma LH-activity is related to hypophysial release of LH, then the results indicate that lactation suppresses this release in the ovariectomized rat. Until a more sensitive assay for LH is available it will not be possible to determine if lactation also decreases plasma LH-activity in the rat with intact ovaries. Taken together with the lowered hypophysial LH content found in ovariectomized lactators (4), it appears that synthesis of LH may also be interfered with by lactation. Lactation, perhaps by the suckling stimulus itself, appears to initiate a neural reflex which inhibits LH synthesis and secretion.

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CONJUGATION OF STEROIDS BY LIVER, KIDNEY AND INTESTINE OF MICE

ABSTRACT

The kidney, liver and small intestine of male CBA mice were incubated *in vitro* with cortisol-4-C^{14}, corticosterone-4-C^{14} and allopregnane-3α-ol-11,20-dione-4-C^{14}. It was found that the kidney did not conjugate cortisol, but rendered water soluble a small quantity of corticosterone. In contrast, 31.8% of the 11-ketotetrahydroprogesterone (29% of which was present as glucuronosides) was converted to a water soluble form. The liver converted to a water soluble form 18.8% of the cortisol, 25.4% of the corticosterone (54.6% as glucuronosides) and 42.3% of 11-ketotetrahydroprogesterone (27.2% as glucuronosides). The small intestine did not form water soluble compounds from the above-mentioned steroids.

The liver has been shown to be the major organ responsible for the formation of water soluble conjugated steroids. Eviscerated and hepatectomized animals (1, 2) do not form water soluble conjugates of corticosteroids. The kidney and small intestine contain phenol sulfokinase, but steroid sulfokinase has been demonstrated only in the liver (3). Recently it has been reported that the kidney can conjugate 17-ketosteroids (etiocholanolane, androsterone) with glucuronic acid (4). The possibility exists that the kidney is capable of conjugating other steroids as glucuronosides.

We have observed that a steroid with "tetrahydro" configuration in ring A was conjugated *in vitro* by the kidney. However, corticosteroids containing the α-β unsaturated A ring are poorly, if at all, conjugated. Small intestine did not conjugate any of the steroids studied.

METHODS

Normal male CBA mice 10 weeks of age with body weights ranging from 20–25 gm. were used as a source of tissue. The animals were sacrificed by cervical dislocation and kidneys, livers and small intestines were removed and placed in iced buffer. The intestinal contents were discarded.

The tissues were finely minced with a razor blade. One gram of tissue was placed in 125 ml. Erlenmeyer flasks with 20 ml. of 0.1 M phosphate buffer (pH 7.4) containing 2.5 μM of TPNH and 2.5 μM of glucose-6-phosphate. Fifty mμM each of cortisol-4-C^{14}, corticosterone-4-C^{14} and allopregnane-3α-ol-11,20-dione-4-C^{14} (11 ketotetrahydroprogesterone-4-C^{14}) were incubated in separate flasks with kidney, liver and small intestine at 37° C for three hours. Reaction was stopped by adding 20 ml. of acetone to each flask and the contents immediately extracted four times with equal volumes of warm acetone (40° C). The acetone extracts were evaporated *in vacuo*. The remaining water residue was then exhaustively extracted with equal volumes of ethyl acetate and the extracts evaporated to dryness *in vacuo*. The residue was transferred with $CHCl_3$/MeOH (1:1) to graduated centrifuge tubes. This fraction contained the free non-conjugated steroids.

Aliquots of both the free steroid and the water fractions were counted in a Packard automatic Tri-Carb liquid scintillation spectrometer. The efficiency was determined for organic and water fractions by using an internal standard of C^{14}-toluene. Using these efficiencies, the counts per minute were converted to disintegrations per minute (DPM) and all subsequent calculations are based on disintegrations per minute.

A β-glucuronidase hydrolysis was performed as follows on the water fractions containing the water soluble steroids, using Sigma Chemical Co. β-glucuronidase. The water fractions were made up to a standard volume of 20 ml. and transferred to a 125 ml. Erlenmeyer flask. Fifty mg. of ethylene-diamine tetraacetic acid (disodium salt), 100 mg. of cysteine, 2 ml. of pH 6.5 phosphate buffer (0.75 M), 2,000 units of β-glucuronidase and 1 drop of chloroform were added to each flask in the order given. The flasks were incubated for 17 hours at 37° C–40° C.

After hydrolysis, the samples were re-extracted with ethyl acetate as before, and the organic and water fractions counted for radioactivity. The percent of water soluble conjugates was calculated by dividing the DPM in the water by the total sample DPM (Free DPM $+H_2O$ DPM). Percent glucuronoside was calculated in the same manner by dividing the DPM in the ethyl acetate by the total sample DPM after β-glucuronidase hydrolysis (Free DPM $+H_2O$ DPM).

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TABLE 1. *In vitro* INCUBATIONS OF STEROIDS WITH VARIOUS TISSUES OF MICE

Tissue	Steroid*	No. of experiments	% water soluble† steroids	% water soluble‡ as glucuronoside
Small intestine	Cortisol[1]	2	0.0	0.0
	Corticosterone[2]	4	0.0	0.0
	11-Ketotetrahydroprogesterone[3]	6	0.0	0.0
Kidney	Cortisol	3	0.0	0.0
	Corticosterone	4	4.96 ±0.45	not detectable
	11-Ketotetrahydroprogesterone	7	31.8 ±1.67	29.4 ±2.03
Liver	Cortisol	2	18.8	not determined
	Corticosterone	5	25.4 ±0.81	54.6 ±0.55
	11-Ketotetrahydroprogesterone	8	42.3 ±2.2	27.2 ±1.20

* 50 mμM of steroid incubated.
† Averages & standard deviations.
‡ Percent of steroid glucuronosides extracted from H_2O soluble steroid fraction after β-glucuronidase hydrolysis.
[1] 4.8×10^4 D.P.M. incubated.
[2] 3.9×10^4 D.P.M. incubated.
[3] 7.2×10^4 D.P.M. incubated.

RESULTS

The data of Table 1 show that the small intestine did not conjugate any of the steroids tested. The kidney, on the other hand, did not conjugate cortisol, but did conjugate 5.0% of the corticosterone. However, the kidney was able to conjugate 11-ketotetrahydroprogesterone at almost the same rate as the liver. The amount of corticosterone conjugated by the kidney was too small to yield evidence for β-glucuronidase hydrolysis. Approximately 30% of 11-ketotetrahydroprogesterone was conjugated as glucuronoside.

The liver conjugated all three compounds, although not to the same extent. Conjugation was the smallest in the case of cortisol and greatest with 11-ketotetrahydroprogesterone. The β-glucuronidase hydrolysis of the 11-ketotetrahydroprogesterone conjugated by the liver indicated that 27% of the conjugates were as glucuronosides.

DISCUSSION

The liver can conjugate a Δ^4-3-ketosteroid because of its ability to reduce the steroid to a tetrahydro compound (reduced in ring A) (5). The formation of water soluble conjugates (5) is a sequel to this reduction. Cortisol is conjugated to a lesser extent than corticosterone, which differs from cortisol by the lack of the 17-OH group; it is interesting to note that the half life in blood is different for these compounds. Cortisol has a longer half life than corticosterone (6).

Compounds reduced in ring A are conjugated to a much greater extent and have a shorter half life than non-reduced compounds (6). Although the amount of tetrahydrosteroid conjugated by the liver is greater than the amount of corticosterone conjugated the percent glucuronoside is greater for corticosterone than for the tetrahydro compound.

The kidney has been reported to conjugate C-19 tetrahydro compounds (4). Our studies show that the kidney can conjugate a C-21 tetrahydro compound. These results suggest that the hydroxy group in position three is necessary for conjugation. The kidney in the presence of TPNH and glucose-6-phosphate is capable of conjugating small quantities of corticosterone. The kidney was able to catalyze conjugation with glucuronic acid in the same degree as the liver from the tetrahydro compound. The kidney in the

presence of TPNH and glucose-6-phosphate was not able to conjugate any measurable quantities of cortisol. It is important to note that the adrenal of mice produces mainly corticosterone.

The small intestine was incapable of conjugating any of the steroids incubated in the presence or absence of cofactors.

It was recently found that the parenchymal cell of the liver is the cell which controls the conjugation of the steroids (6), and that the reticuloendothelial cell has a large capacity to oxidize and reduce the substituted groups of the steroid nucleus (7). Histologically the kidney is mainly a parenchymatous organ with very little reticuloendothelial system. The liver which contains approximately one third (33.4%) reticuloendothelial cells (8) actively reduces ring A to form tetrahydro steroids with subsequent conjugation of the tetrahydro steroids by the hepatic cell (6, 7). The kidney, however, reduces ring A to much lesser extent and hence cannot actively conjugate Δ^4-3-keto compounds, but can conjugate a tetrahydro compound as readily as the liver.

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Acknowledgment

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FORTY-THIRD ANNUAL MEETING OF THE ENDOCRINE SOCIETY

GENERAL INFORMATION:

Date: Thursday, Friday and Saturday
June 22–23–24, 1961

Headquarters: Hotel Biltmore
New York, New York

Registration: Fountain Room, Hotel Biltmore

Everyone attending the meetings is required to register. By action of the Council, non-members will be charged a registration fee of $10.00 for any part of the scientific sessions.

Fellows, graduate students, residents and interns will be admitted upon presentation of proper credentials CERTIFIED BY DEPARTMENT HEAD OR SUPERINTENDENT OF HOSPITAL and payment of $2.00 registration fee.

Members should present their current membership cards when registering. Admission will be by badge only.

Your Endocrine Society badge will admit you to the American Diabetes Association's opening meeting Saturday morning at the Hotel Commodore. Members of the Endocrine Society are requested to register (without charge) at the desk of the American Diabetes Association.

Scientific Sessions: All morning sessions will be held in the Ballroom. Simultaneous sessions will be held each afternoon in the Ballroom and the Bowman Room. Sessions will begin promptly on schedule. Presentation of all submitted papers will be LIMITED TO TEN MINUTES. All speakers will be required to adhere strictly to schedule. Speakers who have manuscripts for publication are invited to submit them to the Editor of ENDOCRINOLOGY or *The Journal of Clinical Endocrinology and Metabolism.*

Annual Dinner: The Annual Dinner of the Society will be held on Friday evening, June 23, at 7:30 o'clock in the Ballroom. Cocktails will precede the dinner in the Fountain Room at 6:30 P.M. Secure tickets at time of registration.

Local Arrangements: Dr. Nicholas P. Christy is Local Chairman and Mrs. Warren O. Nelson is in charge of the Women's Entertainment Committee.

PROGRAM

THURSDAY, JUNE 22, 1961

8:00 A.M.—Registration, Fountain Room, Hotel Biltmore

SESSION I. 9:00 A.M.—PITUITARY

Room: Ballroom

Co-Chairmen: *Roy O. Greep*
Alfred E. Wilhelmi

1. CHROMATOGRAPHY OF CANINE HYPOTHALAMIC EXTRACTS
Andrew V. Schally, Harry S. Lipscomb, John M. Long (by invitation), *Wayne E. Dear* (by invitation), and *Roger Guillemin.*

2. Evidence for Central Nervous System Inhibition of ACTH Secretion
Richard H. Egdahl.
3. Influence of Alterations in Structure of the N-Terminal Serine on Corticotropin Activity
Harold E. Lebovitz (by invitation), *Frank L. Engel, H. B. F. Dixon* (by invitation), and *Lowell R. Weitkamp* (by invitation).
4. Diffuse Hypothalamic System in the Regulation of ACTH Release
A. Brodish.
5. Effects of Reserpine and Hypothalamic Lesions on Pituitary Lactogen Content in the Rabbit
S. Kanematsu (by invitation), *J. Hilliard* (by invitation), and *C. M. Sawyer.*
6. Secretion of Prolactin by Pituitary *in vitro*
Joseph Meites, Raymond H. Kahn, and *C. S. Nicoll* (by invitation).

INTERMISSION

7. Biologic Activities of Human Hypophysial Mammotrophin
Wm. R. Lyons, Choh Hao Li, and *Ruth E. Johnson* (by invitation).
8. Effect of Experimentally-Induced Hypo- and Hyperthyroidism upon Pituitary Lactogenic Hormone Concentration
Clark E. Grosvenor.
9. Immunochemical Studies on Human Growth Hormone in Blood and Urine
Selna L. Kaplan (by invitation), and *Melvin M. Grumbach.*
10. Further Evidence for Amino Acid Transport as a Site of Action of Growth Hormone
E. Knobil and *P. F. Brande* (by invitation).
11. Extraction of Vasopressin by Percolation
Murray Saffran, Brigitte U. Caplan (by invitation), *Seymour Mishkin* (by invitation), and *Brahm Muhlstock* (by invitation).
12. Three Types of Basophils of the Human Adenohypophysis and the Hormones They Produce
Calvin Ezrin and *William C. Nicholas.*

THURSDAY, JUNE 22

SESSION II. 2:00 P.M.—PITUITARY; REPRODUCTION (FEMALE)

Room: Ballroom

Co-Chairmen: *James T. Bradbury*
Melvin M. Grumbach

13. Specific Hypothalamic Sites at Which Progesterone Acts to Facilitate Ovulation in the Rat
Charles A. Barraclough and *S. Yrarrazeval* (by invitation).
14. Sexual Differences in the 24-Hour Rhythm of Pituitary-Adrenal Function in Rats
Vaughn Critchlow, Harry S. Lipscomb, Robert A. Liebelt (by invitation), *Mildred Elwers* (by invitation), and *Walter E. Mountcastle* (by invitation).
15. A New Parameter for Biological Characterization of Gonadotropins: Species Differences and Significance for Bioassay
Albert F. Parlow.
16. Changes in Pituitary LH Content During the Rat Estrous Cycle
Neena B. Schwartz and *Delphine Bartosik* (by invitation).

17. The Time Required for Release of Ovulation-Inducing Hormone from the Rat Hypophysis After Stimulation of the Preoptic Hypothalamus
John W. Everett and *H. M. Radford* (by invitation).
18. A Hypothalamic Luteinizing Hormone (LH)-Releasing Factor
S. M. McCann.

INTERMISSION

19. Effect of Exogenous Human FSH on Ovaries and Ovarian Steroids
Robert B. Greenblatt and *Virendra B. Mahesh* (by invitation).
20. An Electron Microscope Study of the Ovarian Response to Gonadotrophin
James A. Green (by invitation).
21. Gonadal Disorders with Unusual Chromosomal and Karyologic Findings
Avery A. Sandburg, George F. Koepf, and *Thomas F. Frawley.*
22. Importance of Oxidation Reactions in the Initiation of Uterine Growth by Estradiol
E. V. Jensen, J. W. Flesher (by invitation), *N. N. Saha* (by invitation), and *H. I. Jacobson* (by invitation).
23. Radioautographic Studies of the Decidual Reaction in Pseudo-Pregnant Mice Treated with Tritiated-Thymidine
Key June Seoung (by invitation).
24. Enzymes in Rat Uteri During Post-Partum Involution
Bertha L. Lobel (by invitation), and *Helen Wendler Deane.*

THURSDAY, JUNE 22

SESSION III. 2:00 P.M.—ADRENAL

Room: Bowman Room

Co-Chairmen: *William W. Tullner*
Don H. Nelson

25. Fractionation of Crude Kidney Extracts for Aldosterone Stimulating Activity
James O. Davis, Elwood Titus (by invitation), *Herbert E. Spiegel* (by invitation), *Carlos R. Ayers* (by invitation), and *Charles C. J. Carpenter* (by invitation).
26. Effect of Angiotensin and ACTH on Adrenocortical Secretion in Hypophysectomized Nephrectomized Dogs
William F. Ganong, Patrick J. Mulrow, and *Gerald Cera* (by invitation).
27. Aldosterone Biosynthesis: The Influence of Various Precursors, ACTH and Angiotensin II
Norman M. Kaplan, and *Frederic C. Bartter.*
28. Recent Observations on Adrenoglomerulotropin
Gordon Farrell.
29. Synthesis of Aldosterone in Hypertensive Congenital Adrenal Hyperplasia with "Complete" Deficiency of 11β-Hydroxylation
Donald A. Holub and *Joseph J. Barlow* (by invitation).
30. The Turnover Rate, Distribution, and Plasma Binding of Aldosterone in Pregnancy
D. S. Layne (by invitation), *B. Little* (by invitation), *C. J. Meyer* (by invitation), *J. F. Tait,* and *S. A. S. Tait.*

INTERMISSION

31. Studies of Adrenocortical Function of Hirsute Women
Ross D. Jacobs, Julia Lobotsky (by invitation), *Arnold M. Moses, Edward L. Klaiber* (by invitation), and *Charles W. Lloyd.*
32. Impaired Steroid Biosynthesis in Adrenocortical Cancer
Mortimer B. Lipsett, and *Hildegard Wilson.*
33. Renal Unresponsiveness to Salt Retaining Hormones in Infancy
G. Leboeuf (by invitation), *D. D. Steiker* (by invitation), *A. M. Bongiovanni* and *W. R. Eberlein.*
34. Adrenal Inhibitors and Stimulators
Joseph J. Barlow (by invitation), and *Donald A. Holub.*
35. Reduction of Adrenal Steroid Production by an Inhibitor of Cholesterol Biosynthesis (MER-29)
James C. Melby, Sidney L. Dale (by invitation), and *Marlys St. Cyr* (by invitation).
36. Two New Inhibitors of the Adrenal Cortex
J. J. Chart and *H. Sheppard* (by invitation).

FRIDAY, JUNE 23

8:30 A.M.—Registration, Fountain Room

SESSION IV. 9:00 A.M.—REPRODUCTION (MALE; FEMALE)

Room: Ballroom

Co-Chairmen: *E. Perry McCullagh*
Sheldon S. Segal

37. Dichotomous Effect of Bis-(Dichloroacetyl) Diamines upon Spermatogenesis, Leydig-Cell and Gonadotropin Function in Man
Carl G. Heller, Donald J. Moore (by invitation), *Yves Clermont* (by invitation), and *C. Alvin Paulsen.*
38. Urine Estrogen Response to Human Chorionic Gonadotrophin (H.C.G.) in Young, Old and Hypogonad Men
W. I. Morse, Albert Clark (by invitation), *W. A. Ernst* (by invitation), and *C. L. Gosse* (by invitation).
39. Steroidogenesis in the Human Foetal Testes
Hernan F. Acevedo, Leonard R. Axelrod, Eisei Ishikawa (by invitation), and *F. Takaki* (by invitation).
40. Estimation of the Secretory Rate of the Testicular Precursors of Urinary 17-Ketosteroids
Paul C. MacDonald (by invitation), *Raymond L. VandeWiele,* and *Seymour Lieberman.*
41. The Effect of 19-Hydroxyandrostenedione on the Aromatization of Δ4-Androstenedione-3,17
Nina Hollander (by invitation).
42. Further Studies in the Renal Handling of Endogenous Estrogens in Late Pregnancy
C. H. Brown (by invitation), and *John R. K. Preedy.*

INTERMISSION

43. The Use of Bromine-82 in the Analysis of Estrogens
W. R. Slaunwhite, Jr., and *L. Neely* (by invitation).
44. Steroidogenesis in the Polycystic Ovary
Leonard R. Axelrod, and *Joseph W. Goldzieher.*

45. Regulation of Nucleic Acid Synthesis by Androgens
Charles D. Kochakian, and *David G. Harrison* (by invitation).
46. A New Pathway for the Biosynthesis of Testosterone
Enrico Forchielli (by invitation), *Marcel Gut* (by invitation), and *Ralph I. Dorfman*.
47. Stimulation of Incorporation of Acetate into Testosterone by FSH and LH *in vitro*
Peter F. Hall (by invitation), and *Kristen B. Eik-Nes*.
48. Hormonal Factors in the Adrenal-Dependent Growth of the Rat Ventral Prostate
William W. Tullner.

FRIDAY, JUNE 23

1:30 P.M.—ANNUAL BUSINESS MEETING

Room: Ballroom

FRIDAY, JUNE 23

SESSION V. 2:30 P.M.—THYROID

Room: Ballroom

Co-Chairmen: *Joseph Edward Rall*
Samuel P. Asper

49. Detection of Antibodies to Thyroglobulin by Paper Electrophoresis of Serum Containing Thyroglobulin—I^{131}
I. N. Rosenberg, and *F. Pamukcu* (by invitation).
50. Immunologic Properties of Thyrotropin
Richard P. Levy, *William L. McGuire* (by invitation), and *Lawrence Heideman, Jr.*
51. Transplacental Transfer of Thyroid Isoantibodies in the Rabbit
William H. Beierwaltes, *Katherine E. Elzinga* (by invitation), *Robert W. Schmidt* (by invitation), *Vernon N. Dodson* (by invitation), *Norma P. Spafford* (by invitation), and *Edward A. Carr, Jr.*
52. Correlation of Thyroid Hormone Metabolism and Effect in *Necturus maculosus*, a Thyroxine-Resistant Species
Valerie Anne Galton, and *Sidney H. Ingbar*.
53. An Evaluation of the Physiological Role of Prealbumin in the Binding of Thyroxine
C. S. Hollander (by invitation), *V. V. Odak* (by invitation), *T. E. Prout*, and *S. P. Asper, Jr.*
54. Thyroxine-Binding in Human Fetal Serum
M. Andreoli (by invitation), and *J. Robbins*.

INTERMISSION

55. Normal PBI and Decreased Serum Thyroxine-Binding in Two Sisters with Hyperthroidism
R. R. Cavalieri (by invitation).
56. The Interrelationships Between Fatty Acids and TSH Action in Sheep Thyroid Tissue
Norbert Freinkel, and *Edward C. Saef* (by invitation).
57. Effect of Hypophysectomy and TSH on Synthesis and Secretion of Thyroglobulin Precursor in Thyroid Epithelium
N. J. Nadler, and *B. Mitmaker* (by invitation).

58. Urinary Catechol Amines and VMA in Patients with Hyperthyroidism and Hypothyroidism
John G. Wiswell, Gilbert E. Hurwitz (by invitation), Victor Coronho (by invitation), and *Oscar H. L. Bing* (by invitation).
59. Stimulation of Glucose-6-C^{14} Metabolism in Rat Thyroid Slices by Propylthiouracil (10^{-8}M)
Philip F. Mulvey, Jr. (by invitation), and *D. Ward Slingerland.*
60. Inhibition of Thyroidal I^{131}-Utilization *in vitro* by an Intrathyroidal Substance
Winton Tong (by invitation), and *I. L. Chaikoff.*

FRIDAY, JUNE 23

SESSION VI. 2:30 P.M.—CARBOHYDRATE, LIPID, and CALCIUM METABOLISM

Room: Bowman Room

Co-Chairmen: *Leslie L. Bennett*
Francis D. W. Lukens

61. Direct Effect of Insulin on Liver Slices from Depancreatized Frogs
J. C. Penhos (by invitation).
62. Contribution of the Pentose Cycle to Glucose Metabolism in Rat Adipose Tissue *in vitro*
Bernard R. Landau, and *Jack R. Leonards* (by invitation).
63. Effect of Mannoheptulose on Blood Glucose and Ketone Bodies in the Fasting Rat
Robert O. Scow, Ernst Simon (by invitation), and *Sidney S. Chernick* (by invitation).
64. Effect of Hypophysectomy on Metabolic Changes Produced by Growth Hormone in Juvenile Diabetics
Dorothy H. Henneman, Eleanor McGarry, and *John C. Beck.*
65. A Simplified Technique for Measurement of Insulin Binding Globulin
G. J. Dendrinos (by invitation), *T. E. Prout,* and *V. V. Odak* (by invitation).
66. Insulin Resistant Diabetes Associated with Increased Endogenous Plasma Insulin Followed by Remission
James B. Field.

INTERMISSION

67. An Insulin Secretion and Storage Defect in Certain Insulinomas
R. H. Pollen (by invitation), *G. M. Grodsky* (by invitation), and *V. C. DiRaimondo.*
68. Effects of Human Growth Hormone on Lipid Metabolism in Diabetic Subjects
R. E. Visintine (by invitation), *G. D. Michaels* (by invitation), and *L. W. Kinsell.*
69. The Specific Structure of Compounds Effecting Fat Mobilization in Man
Morton D. Bogdonoff, Joseph W. Linhart (by invitation), *Robert F. Klein* (by invitation), and *E. Harvey Estes, Jr.* (by invitation).
70. Effects of Vitamin D and Cortisone on Intestinal Absorption of Calcium in the Rat
Gerald A. Williams, E. Nelson Bowser, and *Walter J. Henderson* (by invitation).
71. Use of Continuous Infusions of Calcium-45 to Study Skeletal Metabolic Function
Clayton Rich.
72. Balance Studies of Calcium Loading in Osteoporosis

Ernest Schwartz (by invitation), *William V. Chokas* (by invitation), and *Richard L. Fogel* (by invitation).

FRIDAY, JUNE 23

SESSION VII.—ANNUAL DINNER, Hotel Biltmore

John E. Howard, President, presiding

6:30 P.M.—Cocktails

Fountain Room
Sponsored by the donors of the Awards of the Society:
Ciba, Ayerst, Upjohn and Squibb
Attendance limited to holders of dinner tickets.

7:30 P.M.—Dinner

Ballroom
Presentation of the 1961 Awards of the Society by
Dr. E. Perry McCullagh:
The Fred Conrad Koch Award
The Ciba Award
Ayerst Fellowship
Upjohn Scholar
President's address: Dr. Howard

SATURDAY, JUNE 24

8:30 A.M.—Registration, Fountain Room
Session VIII. 9:00 A.M.—ADRENAL (Continued)

Room: Ballroom

Co-Chairmen: *Frederic C. Bartter*
Ralph I. Dorfman

73. 6-β-HYDROXY-CORTISOL: ORIGIN IN NORMAL SUBJECTS AND ELEVATION BY ESTROGEN TREATMENT
Marvin M. Lipman (by invitation), *Fred H. Katz* (by invitation), *Andrew G. Frantz* (by invitation), and *Joseph W. Jailer.*

74. ISOLATION AND PROPERTIES OF THE CORTICOSTEROID BINDING GLOBULIN (TRANSCORTIN) FROM NORMAL HUMAN PLASMA
Ulysses S. Seal (by invitation), and *Richard P. Doe.*

75. STUDY OF 17-HYDROXYPROGESTERONE-4-C^{14} IN MAN
David K. Fukushima, H. Leon Bradlow, Leon Hellman, Barnett Zumoff (by invitation), and *T. F. Gallagher.*

76. RATE OF PRODUCTION AND DEGRADATION OF CORTISOL IN OBESITY
J. P. Eckert (by invitation), *O. C. Green* (by invitation), and *C. J. Migeon.*

77. SUPPRESSION OF PITUITARY ACTH RESPONSE IN THE HUMAN BY ACTH ADMINISTRATION
John E. Plager, and *Paul Cushman, Jr.,* (by invitation).

78. STUDIES ON THE 21-HYDROXYLASE AND Δ^5-3β-HYDROXYSTEROID DEHYDROGENASE OF THE HUMAN ADRENAL
Lorna J. Langer, and *Steven M. Podos* (by invitation).

INTERMISSION

79. Hormonal Effects on Distribution of Amino Acid Radioactivity in the Adrenal of the Male Rat
E. D. Bransome, Jr. (by invitation), and *W. J. Reddy.*
80. Glucose Metabolism in the Adrenal Gland
Vernon K. Vance (by invitation), *Francois Girard* (by invitation), and *George F. Cahill, Jr.*
81. Biosynthetic Capabilities of Isolated Adrenal Reticuloendothelial Cells
David L. Berliner, C. J. Nabors, Jr. (by invitation), and *Thomas F. Dougherty.*
82. Isolation of 18-Hydroxycorticosterone from Rat Adrenals
Fernand G. Peron.
83. Effect of Saluretic Agents on Blood Pressure and Thyroid Activity of Hypertensive Rats
Melvin J. Fregly.
84. ACTH Induced Changes in the State of Oxidation of Adrenal Ascorbic Acid
Boyd W. Harding (by invitation), *Elmer R. Rutherford* (by invitation), and *Don H. Nelson.*

SATURDAY, JUNE 24

SESSION IX. 2:00 P.M.—SYMPOSIUM ON PARA-ENDOCRINE DISORDERS

Room: Ballroom

Chairman: *John E. Howard*

2:00–2:20—Derangement of Calcium Metabolism in Sarcoidosis
(See abstract 89).
Norman, H. Bell, John R. Gill, and *Frederic C. Bartter,* National Heart Institute, Bethesda, Md.
DISCUSSION

2:30–2:50—Malignant Tumors Mimicking Hyperparathyroidism
(See abstract 100).
Thomas B. Connor, William C. Thomas, William B. Stodghill, and *John E. Howard,* The Johns Hopkins University and The University of Maryland School of Medicine, Baltimore, Md.
DISCUSSION

3:00–3:30—Severe Cushing's Syndrome Apparently Due to Corticotropin Secreting Oat Cell Carcinoma
(See Abstract 162).
Clifton Meador, Donald Island, Wendell Nicholson, John Nuckton, Charles Lucas, John Luetscher, and *Grant Liddle,* Departments of Medicine, Vanderbilt University and Stanford University, Nashville, Tennessee and Stanford, California.
DISCUSSION

3:30–3:50—Polycythemia Secondary to Neoplasms (No abstract)
Thomas A. Waldmann, National Cancer Institute, Bethesda, Md. (by invitation).
DISCUSSION

4:00–4:20—Disturbances in Carbohydrate Metabolism in Cancer Patients (No abstract)
Paul A. Marks, Columbia College of Physicians and Surgeons, New York City (by invitation).
DISCUSSION

INTERNATIONAL UNION AGAINST CANCER

Eleanor Roosevelt International Cancer Fellowships

The International Union Against Cancer, through funds made available by the Eleanor Roosevelt Cancer Foundation, will award annually fellowships for research on cancer. These fellowships have been created in the belief that the international exchange of scientists between centers with kindred interests will facilitate the sharing of knowledge and thereby contribute to the control of cancer.

These are senior postdoctoral awards designed for the support of persons who, as full-time members of the staff of universities, teaching hospitals, research laboratories or other institutions, have demonstrated interest and outstanding ability or promise as independent investigators in the field of research on basic cancer, its experimental and clinical aspects, and who wish to broaden their experience by a period of study in another country. The duration of the fellowships ordinarily will be one year but this period may be extended or shortened in special circumstances. The stipend will be based on the current salary of the applicant and the salary of persons of comparable qualifications in the place where the applicant expects to study. An allowance will be made for dependents and for costs of travel to and from the fellow's residence and the institution where he will work.

Application forms and additional information may be obtained from International Union Against Cancer, P.O. Box 400, Geneva 2 (Switzerland).

5th PAN AMERICAN CONGRESS OF ENDOCRINOLOGY

The Endocrine Society is offering travel stipends to six young investigators who plan to submit communicated papers to the 5th Panamerican Congress of Endocrinology to be held in Lima, Peru, October 15–21, 1961. Applications for travel grants should be submitted by July 15, 1961 to Dr. Gregory Pincus, Chairman of the Committee for the Endocrine Society, with an abstract of the paper to be presented.

NEUROENDOCRINOLOGY SYMPOSIUM

The Endocrinology Study Section of the National Institutes of Health is sponsoring a Symposium on Neuroendocrinology to be held on December 6, 7, and 8, 1961, at Miami, Florida. Registration: December 5 (noon to 6:00 P.M.). Opening meeting: December 6, 9:00 A.M. Those interested in attending should address requests for room reservations to:

Neuroendocrinology Symposium
The New Everglades Hotel
Miami, Florida.

By special arrangement single rooms will cost $8.00, rooms with twin beds and double occupancy, $12.00. These rates will also prevail for a period before and after the meeting, and the hotel management should be contacted for details. For information on the symposium and for a program contact A. V. Nalbandov, 102 Animal Genetics, University of Illinois, Urbana, Illinois. Total attendance at the meeting will be limited to about 250 persons and reservations for rooms will be accepted by the hotel on a first-come, first-served basis after May 1.

DECEMBER 6, 7, 8, 1961

THE NEW EVERGLADES HOTEL, MIAMI, FLORIDA

1. Prologue
   Speaker: R. T. Hill
2. Hypothalamic afferents (limbic system, reticular formation, afferents from midbrain, cortical afferents)
   Speaker: W. J. H. Nauta
   Discussant: P. D. MacLean
3. Vascular connections between hypothalamus, posterior and anterior pituitary gland
   Speaker: J. Landsmeer
   Discussant: H. Duvernoy
4. Neurohypophyseal secretions and their origin
   Speaker: W. H. Sawyer
   Discussants: S. W. Smith
   T. F. Leveque
5. Central nervous system and the secretion and release of ACTH
   Speaker: W. F. Ganong
   Discussants: R. H. Egdahl
   E. M. Anderson
6. CNS and the secretion and release of TSH
   Speaker: S. A. D'Angelo
   Discussant: G. W. Harris

7. CNS and the secretion and release of LH and FSH
   Speaker: B. Flerkó
   Discussants: C. A. Barraclough
   G. W. Harris
8. CNS and the secretion and release of prolactin
   Speaker: J. Meites
   Discussants: S. J. Folley
   A. S. Parkes
   H. N. Bruce
9. Physiology of the pituitary gland as affected by transplantation or stalk transection
   Speaker: J. W. Everett
   Discussants: R. K. Meyer
   R. Courrier
   V. Critchlow
   A. V. Nalbandov
10. Recent advances in the chemistry of neuroendocrine chemical mediators originating in the CNS
    Speaker: R. Guillemin
    Discussants: J. C. Porter
    S. D. McCann
11. Role of peripheral nerves in the function of gonads, accessories and duct systems
    Speaker: R. M. Melampy
    Discussants: R. T. Hill
    A. V. Nalbandov
12. Role of light on the neuroendocrine system
    Speaker: V. Critchlow
    Discussant: A. Wolfson
13. Pharmacology of neuroendocrine blocking agents
    Speaker: P. L. Munson
    Discussant: C. H. Sawyer
14. Human neuroendocrinology
    Speaker: C. Lloyd
    Discussants: A. E. Rakoff
    I. A. Mirsky
15. Concluding Session: Synthesis and summary of symposium
    Speaker: R. O. Greep